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The Clinic for Thoracic Surgery Central Hospital of University of Helsinki
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FINE STRUCTURE OF THE DOG NEPHRON AFTER PERFUSION WITH DONOR BLOOD IN HYPO AND NORMOTHERMIA

By

E A Laitinen L Telivuo and L Virkkula

Received 12 x 64

Perfusion with donor blood during open heart surgery introduces an excessive amount of foreign protein into the circulation of the patient In experimental animals an increase in large droplets in the tubular epithelium of the kidney has been observed after the injection of egg white (*Oliver* 1948, *Zollinger* 1950, *Rhodin* 1954) According to *Oliver et al* (1954) native or foreign proteins which have passed through the glomerulus are resorbed by the cells in the middle portion of the convoluted tubule where they are concentrated as visible droplets The nature of these resorption droplets was considered by these authors to be a substrate derived from resorbed protein and mitochondrial enzymes

Straus (1956) pointed out that the enzymatic properties of the kidney droplets resembled those of lysosomes, isolated from liver cells by *De Duve et al* (1955) *Straus* (1957) fractionated small and large droplets from the homogenized tissue of the rat kidney and observed a considerable decrease in the number of the small droplets with a simultaneous increase in that of the large droplets in the animals treated with egg white After this treatment also the content of acid phosphatase, ribonuclease, desoxyribonuclease, and cathepsin was significantly decreased in fractions containing the small droplets and increased in the supernatant fluid He concluded that the injection of egg white caused the transformation of small droplets into large droplets when some of the hydrolytic enzymes were concomitantly released

Miller (1960) found in the mouse kidney that ox haemoglobin, administered intraperitoneally, passed through the brush border between the microvilli, entered the channels of tubular invaginations at the bases of the brush border and was transported in bulk into vacuoles in the intermediate cell zone These vacuoles increased in size and became transformed through further concentration into dense absorption droplets He described how 15 hours after injection and later, ferritin

[1] Supported by a grant from Sigrid Jusélius Foundation

and systems of layered membranes become visible in the droplets as their density decreases Within 3 to 4 days the droplets contained aggregates of ferritin in the tubule cells Later on *Thoenes* (1962) reported the presence of the layered membranes in the lipid granules of the epithelium of the normal tubule in the mouse kidney

On the other hand after ligation of the ureter *Novikoff (1959)* described the presence of limited vacuoles containing mitochondria in the epithelial cells of the proximal tubular membrane which also displayed acid phosphatase activity He suggested the name cytolysome for them Vacuoles of a similar kind have been observed by *Novikoff & Essner* (1962) in tubule cells also after intravenous injection of deter gent (Triton WR 1939) and by *Ashford & Porter* (1962) in the liver cells after the perfusion of the isolated liver with glucagon These authors have envisaged these vacuoles as representing pathological changes typical for cells undergoing cytolysis They were also found by *Napolitano* (1963) in the brown adipose cells of the interscapular fat pad of rats starved for 12 hours and then placed for 6 hours in a cold room 5–7° C without food In these vacuoles he described the presence of a varity of adielectronic bodies and in many instances degenerating mitochondria They occurred more frequently in cells nearly devoid of lipid and were not *observed in the brown adipose cells of control* animals In his opinion there is little reason to think that the cells in this study were undergoing cytolysis but it seems most probable that metabolism of these cells had been reoriented so that the rat of lipolysis is significantly greater than the rate of lipogenesis

Since the experimental procedures listed above are able to increase lysosomes and cytolysomes in number we would expect similar changes *in the renal tubule to appear also after the excessive introduction* of foreign protein into the circulation during donor blood perfusion This study included different segments of the nephron in the canine kidney in order to decide whether changes of this kind possibly expressing early phases of necrobiotic cytolysis could result from donor blood perfusion

MATERIAL AND METHODS

Mongrel dogs weighing 9 15 kg were used for experiments The animals were pre medicated with morphine hydrochloride and anaesthetized with thiopentonesodium administered intravenously in 2 5 per cent solution in doses of 20–40 mg/kg of body weight If necessary for the maintenance of anaesthesia additional doses were given Pure oxygen inhaled at positive pressure provided the oxygen supply

To minimize the effect of massive tissue trauma upon the results a arterial by pass perfusion only was performed The extracorporeal flow was undertaken by means of the de Bakey pump of the Melrose NIP heart lung machine 1 litre of heparinized (20 mg/0 5 litre) donor blood stored for an hour was used for perfusion

Three animals were *perfused in hypothermia During the experimental procedure* the pharyngeal oesophageal and rectal temperatures were regularly recorded the body temperature being lowered to 25° C The dogs were kept for an hour at this temperature while perfusion was performed The arterial and venous pressures and ECG were recorded The arterial pressure in the caudal portion of the body was kept above 75 mgHg In addition haematocrit haemoglobin serum sodium and potassium

ions and creatinin were analysed before beginning the perfusion and in addition immediately afterwards and also 3 hours after perfusion ceased Serum pH pCO_2 and standard bicarbonate were tested at random

Three other animals were similarly perfused in normothermia and examined as before

After four days samples of the marrow and cortex of the kidney were taken from all treated cases under thiopentonesodium anaesthesia. Additional control samples were taken from two untreated animals For the light microscope examination the samples were fixed in 10 per cent neutral formalin For the demonstration of lipids, frozen sections were cut from undehydrated fixed tissue The remainder of the fixed tissue was dehydrated in alcohol in an ascending series of concentrations and embedded in paraffin Serial sections were made and stained by the following methods

Weigert van Gieson method for general morphological consideration,
Acrolein Schiff method for protein accumulations in the tubule (*van Duijn* 1961),
Perls method Turbull Blue method and Haematoxylin Lake method for iron deposits
Sudan IV staining of frozen sections for fatty changes,

F n the 1 ·

trations Before embedd ng the ·
araldite a

Siemens Elmiskop I electron microscope

OBSERVATIONS

All animals tolerated the experimental procedure well and exhibited no disturbance in their general condition during the postoperative period, either As seen in Table 1, the results of laboratory tests made after perfusion showed no marked deviation from those made before the procedure

At the time the samples were taken, the kidneys of all animals seemed normal in size, showing no swelling The capsule stripped away from the parenchyma as usual and it was not thickened The colour of th

... en from cases perfused for one hour in normothermia exhibited casts in the lumen of the convoluted tubule by Weigert van Gieson staining With the acrolein-Schiff method these casts ...

... of red blood cells was to be seen in the peritubular capillaries and even in arterioles or venules These methods, however, demonstrated no definite changes in the epithelium of the related segments or of any other segments of the nephron Iron deposits were not demonstrable by the Perl's, Turnbull Blue or Haematoxylin-Lake me-

1·

thod Sudan IV staining revealed local collections of sudanophilic material in the epithelial cells of the convoluted tubule These were found in the controls as well as in the treated cases with no significant difference in their appearance in any group Neither was any difference found in the fine structure of the nephrons examined in different segments in the samples of these groups (Figs 1–5)

TABLE 1
Electrolyte Balance

	Hypothermia (3 cases)			Normothermia (3cases)		
	I	II	III	I	II	III
Sodium	116	128	137	146	148	148
meg/l	141	133	137	150	152	141
	138	136	146	128	126	118
Potassium	4 0	4 2	6 0	5 1	4 8	4 1
meg/l	3 8	3 5	3 9	5 3	4 0	3 5
	2 9	3 0	3 9	4 9	5 0	5 1
Chloride	110	109	91	103	98	109
meg/l	82	103	97	111	107	105
	104	110	116	110	109	109
Creatinine	0 8	0 6	0 8	0 95	1 25	1 4
mg percent	1 0	1 1	0 95	0 65	1 15	0 7
	1 8	0 9	0 9	0 75	1 0	1 1
Haemoglobin	110	109	113	136	129	144
gram/litre	149	136	148	125	133	138
	144	150	154	136	138	142
Haematocrit	35	33	35	44	42	46
percent	46	45	44	40	40	44
	55	40	46	42	42	46

I = Before beginning the perfusion
II = Immediately after the perfusion
III = 3 hours after perfusion ceased

Fig 1

An electron micrograph of a kidney glomerulus from a case which was perfused with donor blood for one hour in normothermia No changes were observed in the fine structure — In the capillary tuft of the glomerulus a thin and fenestrated line of endothelial cells (En) covers the mesangial basement membrane on the inner surface of the capillary wall The typical cytoplasmic organelles and pinocytic vesicles are situated where the cytoplasm forms a thicker layer Alongside Bowman s space (BS) slender processes of epithelial cells (Ep) frequently interdigitating with similar processes form a fenestrated coat on the basement membrane The same system can be seen to line the basement membrane of the neighbouring capillaries thus forming cytoplasmic bridges and recesses into Bowman s space The cytoplasmic organelles are found mainly in the coherent central portion of the cells where the cytoplasm may show a filamentous structure The cells facing the thick homogeneous membrane of Bowman s capsule form brief cytoplasmic processes The mensangial basement membrane of the capillary wall has a homogeneous structure which is denser in the middle zone (Bm) The broad portion of the mesangium exhib ts a similar homogeneity Some cells (Mc) of irregular shape containing cytoplasmic organelles briefly may be embedded in the mesangial matrix × 11 000


Ep
En
Bm
Bs
Mc
Ep

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Potassium meg/l	4 0 3 8 2 9	4 2 3 5 3 0	6 0 3 9 3 9	5 1 5 3 4 9	4 8 4 0 5 0	4 1 3 5 5 1
Chloride meg/l	110 82 104	109 103 110	91 97 116	103 111 110	98 107 109	109 105 109
Creatinine mg percent	0 8 1 0 1 8	0 6 1 1 0 9	0 8 0 95 0 9	0 95 0 65 0 75	1 25 1 15 1 0	1 4 0 7 1 1
Haemoglobin gram/litre	110 149 144	109 136 150	113 148 154	136 125 136	129 133 138	144 138 142
Haematocrit percent	35 46 55	33 45 40	35 44 46	44 40 42	42 40 42	46 44 46

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Since the evaluation of the quantity of any structures on the basis of electron micrographs even in low magnifications is a very indefinite one only a subjective estimate can be given regarding the presence of lysosomes and cytolysomes in the epithelial cells of the convoluted tubule Their numbers varied moderately in different cells (Figs 2–3) However, several lysosomes were usually present in the section plane of the epithelial cells of the proximal tubule in the untreated as well as in the treated cases Cytolysomes (Fig 5) with clearly demonstrable remains of mitochondrial cristae were similarly found in the epithelium of the distal segment both in the controls and in the treated cases (Figs 5 6) We did not find any increase in the amount or difference in the structure of these organelles in samples of different groups

Occasionally transferred mitochondria were present in the lumen of the tubule (Fig 2) They were found also in the control samples This abnormality was most probably due to the preparatory procedure and not to the necrobiotic decomposition of the epithelium of tubule Actual disruption of epithelial cells and transfer of mitochondria into the lumen were at times seen in the electron micrographs They appeared without any simultaneous signs of destruction in the fine structure as a whole

Neither the basement membranes of the Bowman's capsule nor the convoluted tubule showed any change in structure or in thickness in treated animals compared with control samples A narrow, loose network of collagen fibrils was seen only in the periphery of the basement membrane in the convoluted tubule (Fig 2) The fibrils did not show a tendency to increase in number after perfusion

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Fig 2

Th

to vary according to whether the l men

(CO) in the periphery M = mitochondrion × 12 000


Ep
Ly
Co
Bm
2



Fig 4

Epithelium of the thin segment with normal fine structure from a case perfused for one hour in normothermia — The fine structure of the flattened epithelial cells of the thin segment These cells gives rise to only a few short projections into the lumen In the basal portion small indentations of the cell membrane are present

epithelium of the proximal tubule The mitochondria are small in size and few in number Also the content of endoplasmic reticulum is scanty Ribosomes are dispersed throughout the cytoplasm singly or in groups Golgi apparatus is seen in some cross sections of the cells × 11,000



Fig 3

Basal portion of the epithelium of normal appearance in the proximal tubule from a case perfused for one hour in hypothermia Several lysosomes (Ly) exhibiting varying degrees of decomposition of their contents are seen Compare the large vacuoles of the tubular epithelium in Fig 2 with these bodies × 13,000

Our findings in the peculiar, fine structure in different segments of the nephron do not essentially differ from those published by many earlier investigators (*Rhodin* 1954, 1935, 1957, *Pease* 1955, *Ruska et al* 1957, *Bergstrand* 1957, *I atta et al* 1961, *Thoenes* 1961 a b) Thus they are only briefly summarized in the legends relating to the electron micrographs from treated cases

The fine structure of epithelial cells in the thin segment seems to have been very sensitive to preparatory technique in other recent studies also (*Latta* 1961, *Thoenes* 1961, *Rhodin* 1963) In the present study the segment was preserved quite adequately in the course of preparation thus allowing evaluation of its fine structure in detail

DISCUSSION

According to *Morris et al* (1957) renal blood flow is correlated with the systemic blood pressure and thus ischaemic renal damage can be avoided during perfusion when the pump prime and oxygenation are adequate Transfusion with incompatible blood causing massive haemolysis in known to be able to damage any part of the convoluted tubule Generally the degree of haemolysis caused by modern pump oxygenators during perfusion is not sufficient to reach the levels known to give rise to such a change Perfusion with donor blood is not, however, without hazards

Bettleri & Tanka (1960) reported hyperaemic changes in the glomerular epithelium of experimental animals simultaneously with the deepening of hypothermia By using perfusion for 90 minutes in normothermia in cats and dogs *Sarajas & Saure* (1960) were able to produce changes largely corresponding to those of tubular nephrosis They suggested 5 hydroxytryptamine released from disintegrated platelets as a causative agent Recently *Gadboys et al* (1962) reported the appear-

Fig 5

Epithelium of the distal segment of the tubule in a case perfused for an hour in normothermia This also exhibits a normal appearance—As in the epithelium of




Ep
Cl
Er
5

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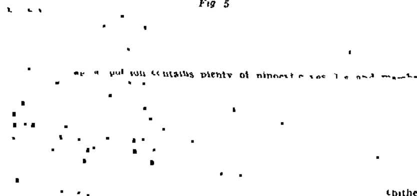
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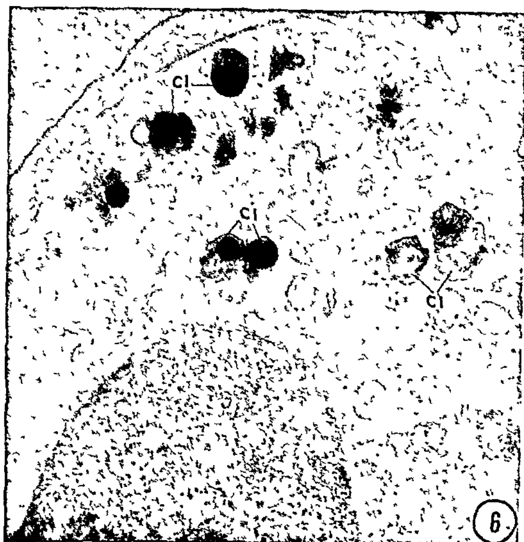


Fig 6

An example of cytolysomes (Cl) with remains of mitochondrial cristae in the *epithelium of the distal tubule of a control case* × 15 000

ance of renal insufficiency in disorder reaction The name "The homologous blood syndrome" was suggested by them and it occasionally appeared in connection with perfusion with homologous donor blood

Our observations are not completely in agreement with those of the above investigators This may be the result of a difference in the experimental technique In emergency conditions is is possible, no doubt, to produce excessive blood trauma which may cause pathological changes in the most sensitive organs The rôle of the extended capillary thrombosis must not be forgotten in this connection

The appearance of casts in the lumen of the convoluted tubule in cases perfused for one hour in normothermia is probably a sign of disturbed kidney function The homogeneous material in the peritubular capillaries, which was occasionally observed in limited areas in these

cases, was possibly the result of local thrombosis Whether this local change is able to induce such a functional alteration, cannot be established with certainty However, the absence of definite changes in the whole fine structure with the simultaneous demonstrability of intra tubular casts in light optic sections is a circumstance suggesting the mechanism of a physiological dysfunction in the production of casts in this experiment Evidently such a disorder is reversible in certain stages *Telkka & Kuusisto* (1958) in a study on the diuretic mechanism of the triazin derivate Orpidan" found the appearance of casts in the tubule with simultaneous engorgement of the epithelium when excessive doses of the medical agent were used This agent depressed the dehydrogenase activity in the epithelium of the proximal convoluted tubules

In the present study we paid special attention to the behaviour of the lysosomes and cytolysomes in the epithelium of the convoluted tubule These structures have exhibited quantitative changes under certain experimental conditions (*Rhodin* 1954, *Novikoff* 1959, *Farquhar & Palade* 1960, *Miller* 1960, *Trump* 1961, *Andersson & Recont* 1962, *Ashford & Porter* 1962, *Napolitano* 1963, *Hruben et al* 1963) In the opinion of *Novikoff* (1963) the release of the hydrolytic enzymes in tearing of the lysosomes may induce the necrobiotic cytolysis

The introduction of an excessive amount of foreign donor blood by perfusion did not obviously effect changes in the amount and structure of these organelles in the tubular epithelium Possibly the tubular epithelium is able within moderate limits to accomodate its capacity for decomposition to an overload of resorbed substances It must be *pointed out that* cytolysomes were demonstrable in the epithelial cells of the distal tubule in control samples too Thus their significance in the evaluation of cellular pathology remains uncertain

SUMMARY

A study was made of the *dog* kidney four days after donor blood perfusion performed in hypo- and normothermia for one hour Different segments of the *nephron* in three cases from both [illegible] were examined [illegible] samples were [illegible] sections taken [illegible] for one hour in normothermia exhibited casts in the lumen of the *convoluted tubule* Simultaneous[illegible] material [illegible] be seen [illegible] lo [illegible] in cases



Fig 6

An example of cytolysomes (Cl) with remains of mitochondrial cristae in the epithelium of the distal tubule of a control case. × 15,000

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Straus W Concentration of acid phosphatase ribonuclease, desoxyribonuclease, B-glucuronidase and cathepsin in "droplets" isolated from the kidney cells of [illegible]

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Rusk tubule

Saraj extra

the realm of other aspects of this disease On the basis of a single lymphnode biopsy it is often impossible to evaluate the predominant histological features Sometimes the diagnosis is based on the examination of a superficial lymphnode alone which allows no conclusion as to appearance of other deeper nodes or lymphnodes at different anatomical sites The lymphnodes may even present a variable picture within their own structure

If however the diagnostic material of a large series were examined a certain trend might be expected to emerge if existing Furthermore if subsequently a postmortem examination were performed the predominant histological presentation might be evident and the question of a correlation with the length of survival if any might be solved

MATERIAL AND METHODS

The h

[illegible] e diagnosis In 2[illegible] cases 8–12 months elapsed before a final diagnosis was made With more than a one year interval between apparent onset of disease and establishment of th[illegible]
was excluded (1) Also excluded wer[illegible]
developed (2) and cases the *autopsy*
causes other than Hodgkin s granulom[illegible]

[illegible] have been
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Acta path et microbiol scandinav 64, 16–30, 1965

The Department of Pathology, Karolinska Sjukhuset, Director Professor B Thorell, and the Department of Radiopathology, Karolinska Sjukhuset, Director Professor Lars Santesson, Stockholm, Sweden

PROGNOSTIC SIGNIFICANCE OF HISTOPATHOLOGY IN HODGKIN'S GRANULOMA

By

HERMAN J LOHMANN

Received 6 vii 64

Jackson & Parker (1947) claim no originality for subdividing Hodgkin's disease into 3 tubtypes They are, however, the best known authors placing special emphasis on the differentiated histological appearance and its prognostic implication in Hodgkin's paragranuloma, Hodgkin's granuloma and Hodgkin's sarcoma The usefullness of this classification has been confirmed by subsequent investigators, most recently by *Smetana & Cohen* (1956) Hodgkin's paragranuloma is characterized by Reed-Sternberg cells against a fairly uniform "background" of lymphocytes Its clinical course can extend over 20 years and more and for this reason it is often referred to as the "benign" Hodgkin Conversely Hodgkin's sarcoma has in an ominously short course Patients rarely survive for periods longer than 2 years, the average survival being less than 1 year The diagnosis is made in the presence of characteristic Reed-Sternberg cells against a "background" of neoplastic reticulum cells

The clinical course as well as the histopathology in Hodgkin's granuloma is not so distinctive The histological picture is by far more bizarre and shows many variants The clinical course can be either of long duration, as in Hodgkin's paragranuloma, or short, as in Hodgkin's sarcoma The mean survival is approximately 30 months with present day treatment Since the histological picture can be so variable, one could suspect a correlation between the histological variant and the clinical course Therefore it becomes of importance to elucidate whether the pathologist might be able to establish such prognostic significance on the basis of features like pronounced eosinophilia, abundant lymphocytes or reticulum cells, fibrosis etc

It would be less than candid, however, not to mention the difficulties involved in such an undertaking The abscence of any major publications in the literature on this particular subject is probably due to these obstacles Only few cursory comments are to be found, usually within

The author wishes to express his appreciation to Professor *B Thorell* Professor *Lars Santesson* and Associate Professor *Bengt Lagerlöf* for their advice and support during the study



Figs 1 2

Fig 1 28 year-old female The marked proliferation of reticulum cells and histiocytes has led to almost complete elimination of granulomatous elements and lymphocytes *Rating* Reticulumcells 3 lymphocytes 1 eosinophiles 2 Survival 20 months Htx eosin × 500

Fig 2 72 year old male Marked proliferation of Reticulum calls with cytologic features of malignancy encroaching on a granulomatous focus This is an example *of how difficult a rigid* classification can be which is often not necessary The presence of granulomatous elements and the eosinophilia was interpreted as *presumptive* evidence for transition into Hodgkin's sarcoma No sequential biopsy Reticulumcells 4 lymphocytes 1 eosinephiles 1, survival 5 months Htx eosin × 400

may not be necessary in some cell elements Reed Sternberg cell for instance will reach class 4 proportions only in extremely rare cases Nevertheless it seemed practicable to use this grading for all histological components for reasons of simplicity and because it was found during the investigation, to give an account of the predominance or scarcity of cell components readily and with sufficient accuracy Approximately the same adenogram can be obtained on the same material by different observers and variations are of such minor degree as to be insignificant for an over all judgement and evaluation Since other aspects of this disease are discussed in numerous publications this analysis is focussed almost entirely on the histopathology and its prognostic significance The histological analysis was done without prior knowledge of the clinical course The examination was for the most part limited to lymphnodes, usually at least two or more Only in rare cases was one large node accepted as representative Following the classification as to cell population, all patients were subdivised into 4 groups according to length of survival patients surviving for less than 2 years, patients living for from 2 years up to 38 months (average survival), from 39 months to 5 years and longer than 5 years

18 of the 147 patients have a history of some radiotherapy prior to obtaining this biopsy material, whether to the area from which the lymphnodes were taken, could not be established with certainty but appears probable, at least in some cases

TABLE 1

50 Cases Surviving Less than 2 Years

Grading	0	1	2	3	4
Lymphocytes		39	9	2	
Reed Sternberg cells		45	5		
Reticulum cells		10	21	15	4
Lymphocytes		39	9	2	
Histiocytis		7	22	21	
Eosinophiles	8	30	9	3	
Plasma cells		32	17	1	
Fibrosis	10	38	2		
Necrosis	5	44	1		
Mitosis	31	14	5		

Grading 0 indicates that these cells or this feature were not identified or to only a minimal degree after long search, 1 implies that these cells were present only inconspicuously, 2 these cells were fairly abundant representing approximately 1/3 to less than 50 per cent of the entire cell population, 3 approximately 50 to 70 per cent make up the histology 4 indicates a a predominance of this cell element, above 75 per cent of the population

RESULTS

A – *Survival Period of Less than 2 Years*

50 patients (33 per cent) survived for less than 2 years or an average of 13 months Average age 37 years 40 of these (80 per cent) show a rating of 2 or higher for reticulum cells, while only 11 (21 per cent) are classed 2 or higher for lymphocytes For histiocytes the rating is even more conspicuous (86 per cent are rated 2 or 3) (Table 1) It is apparent that some of the features which were originally evaluated have not been included in the table, like the follicular or sinusoidal architecture, capsular invasion, epithelioid cells and neutrophils because of their seemingly little significance for prognostic evaluation This may apply also to some of the criteria included, but to a lesser degree It is obvious from this table, that proliferation of reticulum cells and or their histiocytic derivatives appears to imply a markedly shorter survival and there-



Figs 3 4

Fg 3 Same case as F g 2 Showing Reed Sternberg cell with two nucleoli and

Fg 4

fore carries the connotation of an increased clinical malignancy in the absence of the usual criteria for histologically malignant cells The morphological composition is variable, but none of the other cell components seem to be of such importance in this group with short survival The inflammatory cells are pronounced, but in some instances the proliferation of the reticulum cells is so marked that the granulomatous elements are nearly completely eliminated Mitoses are frequent in a number of cases

TABLE 2

32 Cases with Survival between 24 and 38 Months

Grading	0	1	2	3	4
Reed Sternberg cells		25	6	1	
Reticulum cells ,		10	14	8	
Lymphocytes		14	13	5	
Histiocytes		10	15	7	
Eosinophiles	5	20	6	1	
Plasma cells		19	13		
Fibrosis	8	24			
Necrosis	9	23			
Mitosis	20	12			

Grading see Table 1

B – *Survival Period Covering more than 2 Years but Less than the Average Duration of Disease*

This group of Patients (32) survived for more than 2 years but for a shorter time than the overall average of 38 months (Table 2) The average age of these patients was 37 years, the mean survival time being 28 months 22 cases (68 per cent) in this group present a rating of 2 or 3 for reticulum cells and histiocytes Predominance of lymphocytes, however, is not insignificant, 18 cases (56 per cent) are classed 2 or 3 An essential feature which was observed in this group, although not readily evident from the table, is the gradual increase of the granulomatous elements More often than in the other groups the bulk of the lesion consists of inflammatory cells

TABLE 3

33 Cases with Survival from 39 to 60 Months

Grading	0	1	2	3	4
Reed Sternberg cells		31	2		
Reticulum cells		27	6		
Lymphocytes		3	14	15	1
Histiocytes		13	15	5	
Eosinophiles	4	23	6		
Plasma cells		21	10	2	
Fibrosis	8	21	3	1	
Necrosis	9	24			
Mitosis	25	8			

Grading see Table 1



Figs 3 4

Fig 3 Same case as Fig 2 Showing Reed Sternberg cell with two nucleol and d stinct and sharply demarcated nuclear membr n

F g 4 29 year old male

C – *Survival for from 38 to 60 Months (Table 3)*

33 patients survived for a longer period than the average of 38 months but less than 5 years The average age of these patients was 34 years, the mean survival time being 48 months 6 patients (18 per cent) have *a reticulum cell rating* of 2, all others of 1 Histiocytes were classed 2 or 3 in 20 cases (60 per cent) The lymphocytic rating was 2 or higher in 30 patients (90 per cent). Note the relative increase in inflammatory cells !

TABLE 4

32 Cases with more than 5 Years Survival

Grading	0	1	2	3	4
Reed Sternberg cells		27	5		
Reticulum cells		26	4	2	
Lymphocytes		3	2	22	5
Histiocytes		20	12		
Eosinophiles	6	22	3	1	
Fibrosis	6	14	7	5	
Necrosis	20	8	4		
Mitosis	20	11	1		

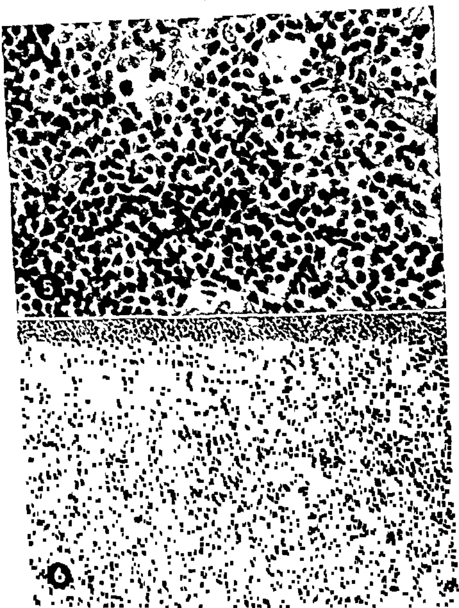
Grading see Table 1

D – *Survival Period Covering more than 5 Years (Table 4)*

32 cases survived for more than 5 years, or on an average 7 years and 5 months Only 4 patients lived for more than 10 years The longest survival time was 12 years and 4 months for Hodgkin's granuloma, the case, starting as paragranuloma and terminating as granuloma, survived for 14 years and 4 months Four of the patients in this group had a lymphocytic rating of 1 or 2, all others 3 or higher At the same time there is a relative scarcity of reticulum cells or their histiocytic derivatives Only 2 patients had a reticulum cell rating of 3, 4 of 2, all others of 1 The histiocytic rating was 2, or 1 (Table 4) Lymphocytic infiltration appears to be the dominant host reaction in this group, however, the granulomatous elements are not insignificant Another essential feature in this group is fibrosis, which has frequently been reported in the literature as a favorable host response, if it is of the so-called spontaneous type and not due to radiation

Evaluation of post mortem Material

In 37 cases of this series it was possible to examine the initial diagnostic material as well as the post mortem material Only minor changes were noted if the time interval was short, marked variation is incurred if the time interval was long The differences noted have a marked relationship to the duration of the disease The lymphocytic background was usually remarkably decreased in the autopsy material Reticulum cell and histiocytic proliferation is present in most cases even though radiation therapy appears to have had an inhibiting effect on this



Figs 5–6

Fig 5 46 year old male Cellular proliferation composed mainly of mature lymphocytes Reticulumcells 1 lymphocytes 4 eosinaphilia 1 Survival 65 months Htx eosin × 400

Fig 6 34 year-old female Predominance of lymphocytes Reticulum cells 1 lymphocytes 3 Survival 49 months Htx eosin × 300

C – *Survival for from 38 to 60 Months (Table 3).*

33 patients survived for a longer period than the average of 38 months but less than 5 years The average age of these patients was 34 years, the mean survival time being 48 months 6 patients (18 per cent) have a reticlum cell rating of 2, all others of 1 Histiocytes were classed 2 or 3 in 20 cases (60 per cent) The lymphocytic rating was 2 or higher in 30 patients (90 per cent) Note the relative increase in inflammatory cells!

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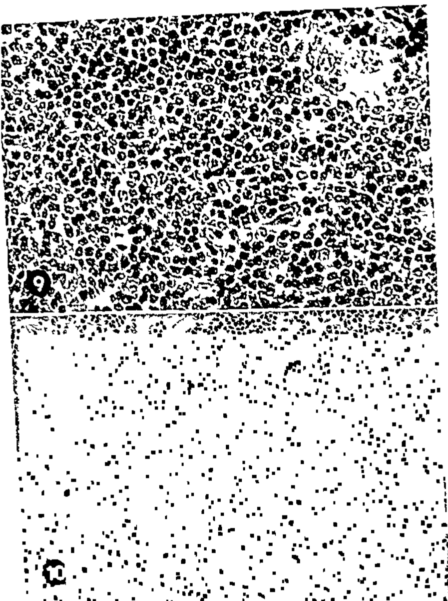
Grading see Table 1

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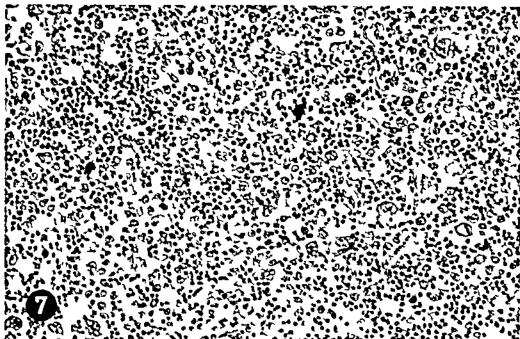
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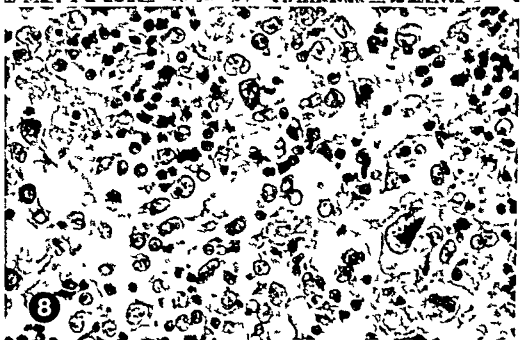


Figs 9–10

Fig 9 51 year old male Cellular population consists mainly of mature lympho cytes Scattered throughout the node are Reed Sternberg cells and atypical histiocytes No eosinophilia Diagnosed as Hodgkin s paragranuloma Lymphocytes 4 Survival 14 years 4 months Htx eosin × 500

Fig 10 Same case as in Fig 9 Sequential biopsy 14 years later The bulk of the lesion consists of inflammatory cells, which have replaced the lymphocytes background Transition of Hodgkin s paragranuloma into Hodgkin's granu loma





Figs 7–8

Fig 7 approximately 3 years oliferation of retic il im ced lymphocytes Htx e sin × 450

Fig 8 Same sli le as Fig 7 With more deta l Htx eosin × 600

detailed analysis of the therapeutic measures used is difficult since many patients were treated in other institutions durong some period of their disease Some general statements can be made however All patients received radiation as the main form of treatment The minimal total dosis was 2000 r Chemotherapy was used in some cases starting in the late 1940 s mostly in form of nitrogen mustard or other alkylating compounds and usually during the final stages of the disease

c) *Clinical Stage and Mode of Dissemination*

The clinical stage is a factor of great prognostic significance *Jackson & Parker* (1947) reported on the basis of an autopsy material that the retroperitoneal and paraaortic lymphnodes were the most frequent primary sites In our series only 15 patients presented the clinical stages II or III using *Peter s & Middlemiss* (1958) clinical staging and none lived longer than the over all average of 38 months This small number is considerably lower than figures found in other series and certainly mislead ng and inaccurate taking into consideration the fact that in ternal lymphnodes so frequently are involved even if not suspected clinically Of interest would be whether the relative shorter survival is reflected in the histopathalogy Many of the patients showed histological features similar to findings in the group of patients who survived for less than 2 years The number of patients however is too small to allow definite conclusions and further study will be necessary

The mode of dissemination is of the utmost importance Involvement of vital organs such as the lungs implies invariably an unfavorable prognosis regardless of the histopathology Furthermore unpredictable changes occur sometimes dramatically in Hodgkin s granuloma These may be sudden remissions as well as exacerbations We do not know whether these sudden changes are reflected in the histopathology *Sequential* biopsies during these periods would be of interest

d) *Age of the Process*

In t

u a ion in *many* cases this time interval is suffici ently long to produce a histopathological picture suited for prognostic evaluation The observation by some authors that eosinophilia is char acteristic of the early stages could not be confirmed in this study A comparison of biopsy and autopsy materials showed no significant difference in the degree of eosinophilia

For these reasons the question of prognosis on the basis of histo pathology can often not be answered with a comfortable degree of assurance Nevertheless the concept of *a gradual* increase in malignancy remains valid for all subgroups of Hodgkin s disease and is expressed

process During the end stages of the disease radiation treatment is usually quite intensive, and this is reflected in most of the autopsy material significant necrosis, fibrosis and marked infiltration by plasma cells and neutrophiles Sequential biopsies, if obtained after a significant time interval, often reflected the same event decrease of the lymphocytic background with increasing prominence of reticulum cells and histiocytes and/or granulomatous cell population

DISCUSSION

The four groups present each characteristic features in their histopathology which appear to be related to the differences in length of survival The question whether there are any criteria in Hodgkin's granuloma by which one can foretell the course of the disease can therefore, according to the findings in this study, be answered in the affirmative This conclusion, however, requires qualification, because of several aspects which must be considered when interpreting the findings in this study

a) *Individual Variation of Histopathology*

In Hodgkin's granuloma the histological picture in the nodes of different regions or even in the different nodes of the same region may vary This has been emphasized in the introduction and is in contrast to Hodgkin's paragranuloma and Hodgkin's sarcoma which generally present a fairly uniform histopathology, even if the material is obtained from different anatomical sites This variation is a hazard in the evaluation of prognostic histological features and limits its value for clinical application Therefore the criteria laid down in this study do not carry such a relative reliable and dependable prognostic implication as the histopathology in Hodgkin's paragranuloma and Hodgkin's sarcoma Furthermore, it is difficult, if not impossible, to predict any changes which may occur suddenly The criteria appear nevertheless useful, since the variation in one node or in lymphnodes from different anatomical sites is not so marked as is generally believed and as was anticipated before this study was commenced Variation in histological presentation does occur, however, but to a considerable extent it is due to cell elements or features like degree of eosinophilia or necrosis, number of Reed-Sternberg cells etc , criteria which seem not so essential for prognostic interpretation

b) *Therapy*

The more healthy and constitutionally strong individual will tolerate and therefore usually receive more treatment than the less healthy patient One can naturally assume that this would tend to create an artefact on survival time not necessarily related to histopathology A

detailed analysis of the therapeutic measures used is difficult since many patients were treated in other institutions durong some period of their disease Some general statements can be made, however All patients received radiation as the main form of treatment The minimal total dosis was 2000 r Chemotherapy was used in some cases starting in the late 1940 s, mostly in form of nitrogen mustard or other alkylating compounds, and usually during the final stages of the disease

c) *Clinical Stage and Mode of Dissemination*

The clinical stage is a factor of great prognostic significance *Jackson & Parker* (1947) reported on the basis of an autopsy material that the retroperitoneal and paraaortic lymphnodes were the most frequent primary sites In our series only 15 patients presented the clinical stages II or III using *Peter s & Middlemiss'* (1958) clinical staging and none lived longer than the over all average of 38 months This small number is considerably lower than figures found in other series and certainly misleading and inaccurate taking into consideration the fact that internal lymphnodes so frequently are involved, even if not suspected clinically Of interest would be whether the relative shorter survival is [illegible]

[illegible] mode of dissemination is of the utmost importance Involvement of vital organs such as the lungs implies invariably an unfavorable prognosis, regardless of the histopathology Furthermore, unpredictable changes occur sometimes dramatically in Hodgkin's granuloma These may be sudden remissions as well as exacerbations We do not know whether these sudden changes are reflected in the histopathology Sequential biopsies during these periods would be of interest

d) *Age of the Process*

In the early stages of the disease lymphocytes [illegible]

[illegible] in many cases this time interval is suffi-
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evaluatic [illegible]
acteristic [illegible]
compari [illegible] and autopsy materials showed no significant difference in the degree of eosinophilia

For these reasons the question of prognosis on the basis of histopathology can often not be answered with a comfortable degree of assurance Nevertheless the concept of a gradual increase in malignancy remains valid for all subgroups of Hodgkin's disease and is expressed

histologically by prevalence or scarcity of certain members of the cell population in the diseased lymphnodes The predominantly lymphocytic background seen in Hodgkin's paragranuloma presents itself as a significant benign factor when encountered in a Hodgkin's granuloma On the other hand, proliferation of reticulum cells and/or their histiocytic derivatives in Hodgkin's granuloma often heralds the ominous clinical course so well known for Hodgkin's sarcoma, even if these cells do not exhibit features of histological malignancy Hodgkin's granuloma therefore, might be considered as the intermediate form of Hodgkin's disease, in many cases capable of showing features of paragranuloma or Hodgkin's sarcoma, depending on the histopathology Abundance of lymphocytes has been noted as a favorable factor in other malignant processes, for instance in the Rous sarcoma and in the medullary carcinoma of the breast The importance of the lymphocytes in the immune mechanism offers interesting aetiological considerations The histopathology could be an expression of the immunological status of the host, but as far as we know no convincing evidence has been presented in the literature that the immune mechanism does not function properly in Hodgkin's disease

Transition of one Type of Hodgkin's Disease into Another

Transformation of a Hodgkin's paragrauloma into Hodgkin's granuloma was observed in one case The first lymphnode biopsy was obtained from the groin, presenting the characteristic appearance of small lymphocytes and Reed-Sternberg cells 14 years later the sequential biopsy obtained from neck revealed the characteristic Hodgkin's granuloma The transformation of a Hodgkin's paragranuloma is a rare occurrence, although it has been demonstrated in some cases

In the 3 cases with sarcomatous proliferation of reticulum cells, sequential biopsies were not available The reticulum cells were approximately 3 times as large as normal lymphocytes and presented a single round or ovoid nucleus with prominent nucleolus *Jackson & Parker* (1947) believed that such cells are undifferentiated forms of the Reed Sternberg cells The clinical course in all three patients was short, less than 20 months, and perhaps they would best be classified as Hodgkin's *sarcoma However, the concomitant presence of unequivocal features* of Hodgkin's granuloma, like marked eosinophilia, plasma cells and neutrophilic leucocytes, in strongly suggestive of transformation from Hodgkin's granuloma into Hodgkin's sarcoma, even without sequential biopsies This is the reason for their inclusion

Significance of Various Forms of Reed Sternberg Cells

While there is little doubt that the Reed-Sternberg cell is one of the neoplastic elements in Hodgkin's disease, it is difficult to demonstrate that the variable forms have a different inherent malignancy "Mature"

Reed Sternberg cells forming multinucleated giant cells are often considered the end stage in the development to a malignant reticuloendothelial cell with cancerous properties This process is sometimes called "Sternbergisation", especially in the German literature Other features interpreted as signs of 'maturity' are (a) an abnormally large nucleolus which shows also vacuolization, and (b) lobation of the nucleus to such a degree that, eventually, part of the nucleus is expelled into the cytoplasma These types of Reed Sternberg cells were seen in the autopsy material, however, one needs only to consider the heavy doses of radiation, and one will exercise extreme caution in the interpretation of these features

Sex and Age of Patients

42 patients were females, a ratio of 3 5 to 1 in favour of males The mean survival of all patients was 38 9 months The mean survival rate is greatests in the age groups from 10–40 years, moderately decreased [illegible] decade We had only [illegible] months There were [illegible] third of all patients in this series survived for less than 2 yeasr and 50 per cent of these patients surviving for short periods were 50 years of age, or older This high mortality among the older patients gains special significance when [illegible]

[illegible] and this change is reflected in the histopathology The shorter than average survival in the older age group can therefore not solely be *attributed* to factors other than Hodgkin's disease This view gains in significance when considering the special effort made to exclude any case with clinical or autopsy evidence of intercurrent disease or other factors contributing to the patients death

SUMMARY

147 cases of Hodgkin's granuloma have been evaluated on the basis of histopathology and correlated with the clinical course A correlation exists apparently between the histopathological presentation and the length of survival The *study has yielded* evidence that there is a gradual increase in inherent malignancy, reflected histologically *in the prevalence* or scarcity of certain members of the cell population in the diseased lymphnodes The predominantly lymphocytic background often signals a relatively favourable prognosis Significantly less favourable is a pronounced granulomatous [illegible]

[illegible] Hodgkin's para[illegible] into Hodgkin's granuloma was demonstrated by sequential

biopsy In 3 cases of Hodgkin's granuloma the proliferative reticulum cells exhibited features of histological malignancy suggestive of transition into Hodgkin's sarcoma, although not demonstrable with sequential biopsies

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THE TWO HISTOLOGICAL MAIN TYPES OF GASTRIC CARCINOMA DIFFUSE AND SO CALLED INTESTINAL-TYPE CARCINOMA

An Attempt at a Histo Clinical Classification

By

PEKKA LAURÉN[1]

Received 19 i 65

The histological classification of gastric carcinomas is difficult as these tumours appear to be very varying structurally This has led to considerable confusion in the histological terminology of gastric cancer Moreover the descriptive histological types such as adenocarcinoma, papillary, solid, scirrhous and colloid carcinoma appear to show a fairly poor correlation with the other features of the disease The significance of histological classification was denied completely by *Stout* (1953) and *Ackerman & del Regato* (1962) who advanced the view that the histological structure of gastric carcinoma shows arbitrary differences in the various parts of the tumour But the necessity of defining the histological basic types associated with the other features of gastric carcinomas has also been emphasized (*Schindler* 1941, *Hampert* 1956 conclusions of the *Symposium on the Geographical Pathology of Gastro intestinal Cancer* 1961)

As gastric carcinoma obviously may be preceded by different pathological changes in the mucosa, and as the gastric mucosa is composed of many different cell types, there is reason to assume that the group of gastric carcinomas also includes forms with specific structural differences In their study of this point *Jarvi & Lauren* (1951) established that the histological structure of gastric carcinoma often displays features characteristic of intestinal mucosa and claimed that in at least 50 per cent of the cases gastric carcinomas arise from intestinal metaplasias in the stomach On the basis of corresponding observations (*Mulligan & Rember* 1954, *Morson* 1955, 1962, *Wattenberg* 1959, *Henschen* 1960) the name ' intestinal type gastric carcinoma has become increasingly used in the literature

The present study—a preliminary report of the results has been

[1] The work was supported by the Damon Runyon Memorial Fund

given earlier (*Lauren* 1964)—tried to establish whether intestinal-type gastric carcinoma constitutes a uniform structural and pathogenetic entity which can be differentiated distinctly from other gastric carcinomas and, whether other histologic carcinoma groups with specific properties exist

Specimens taken at operation for gastric carcinoma on 1344 patients were studied in the Department of Pathological Anatomy, University of Turku in 1945–1964 The specimens were fixed in formalin and studied in sections stained by the haematoxylin-van Gieson technique In addition 309 cases were studied by several other methods Besides the haematoxylin van Gieson technique, Heidenhain's azan technique was used as a general staining method since it displays more clearly the brush border, Paneth's cells and mucus For mucus staining, periodic acid Schiff (PAS) technique after diastase treatment was used, for the demonstration of acid mucus, also alcian blue aldehyde fuchsin and mucicarmine, and for the demonstration of neutral mucus, Best's carmine after diastase treatment was used Both the Masson Hamperl and the Bodian reactions were used to bring out the enterochromaffin cells

The tumours were first classified into groups according to their morphologic characteristics The same tumour often revealed several of these sub-types which corresponded to the descriptive histological types in general use Finally, when the sub-types that were found to be mutually transformable were combined, two main types could be discerned In the series 53 per cent of the tumours (715 cases) were found to belong to a main type consisting mainly of tumours of adenomatous structure The name *intestinal-type gastric carcinoma* already mentioned above was used for this group because all tumours of this structural type occur as primary colon cancer as well Another main type, 33 per cent (441 cases), differed from the intestinal type both in general and cellular structure and in mode of secretion These tumours were called *diffuse gastric carcinoma* in view of their manner of growth In the remaining 14 per cent (188 cases), the structure of the carcinoma differed from both main types This group—a more detailed structural analysis is outside the scope of the present study—is heterogeneous in composition Belonging to this group are carcinomas of intestinal and diffuse type the classification of which involves some uncertainty because of their atypical or poorly differentiated structure A "colliding carcinoma" (*Oota & Tanaka* 1952) of obviously multicentric origin was encountered in a few cases Finally, the group includes tumours of a specific, individual structural type including rare acinar carcinomas provided with excretory ducts which have been reported earlier (*Jarvi & Lauren* 1964)

Neither of the two main types has a direct counterpart among the classical descriptive types of gastric carcinoma Intestinal type carcinomas include, besides pure adenocarcinomas, forms which occur as their variants and can differ considerably from one another in their general structure, but which show similarities both of cellular structure and mode of growth Such sub types are papillary carcinomas and

some solid scirrhous and colloid carcinomas To the diffuse type be long primarily the so called undifferentiated carcinomas and some of the carcinomas earlier diagnosed as solid scirrhous or colloid They can also display a slight tendency to form glandular structure

An endeavour was made to establish how far these two histological main types differ mutually in 1) cell structure with special reference to the presence of intestinal epithelial characteristics 2) mode of secretion and the histochemical properties of the secreta 3) mode of growth and 4) clinical features of the disease

DIFFERENTIAL DIAGNOSIS OF INTESTINAL TYPE AND DIFFUSE GASTRIC CARCINOMA

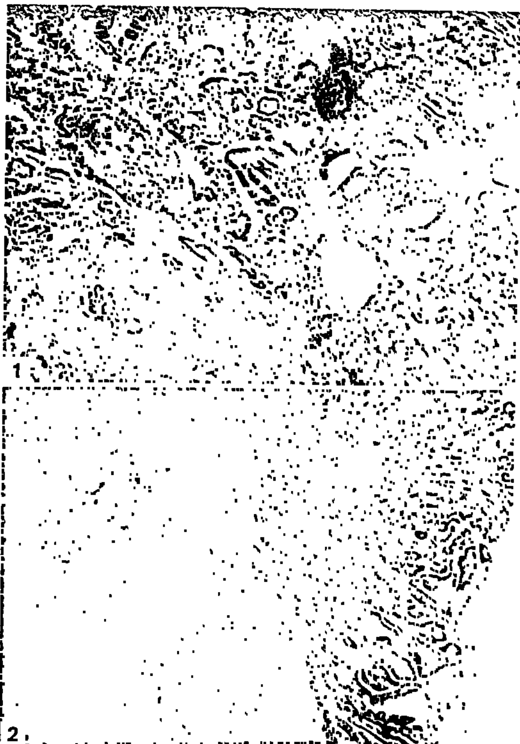
In the classification of the tumours attention was paid to the structural characteristics enumerated below They were of different degree in different tumours and occasionally some of these properties failed to emerge at all

General structure Distinct usually large glandular lumina were typical of intestinal type carcinoma (Figs 1 and 3) They were often accompanied by papillary fold formation or solid components but the glandular structure was rarely completely absent When it was the solid carcinomatous tissue was seen to form distinct epithelial tracts In diffuse carcinomas cells were found to be scattered either as solitary cells or as small clusters of cells (Figs 2 and 4) Glandular lumina were rarely seen when present they were generally quite small and of indistinct shape easy to distinguish from the well defined lumina of intestinal type carcinoma (Fig 5) Diffuse carcinoma occasionally of a more cellular appearance might form apparently solid masses but even in these cases the cells seemed to be only loosely attached to one another forming no distinct epithelium like cords

Cell structure The cells of intestinal type carcinoma were considerably larger more clearly defined and morphologically more variable than the cells seen in diffuse carcinoma In diffuse carcinoma the cytoplasm was indistinctly defined and fragile and the shape of the cells more uniform They were often difficult to recognize as epithelial cells when they appeared as solitary cells or in small clusters

A similar difference was seen in the structure of the nuclei In the intestinal type carcinomas the nuclei were large of variable shape hyperchromatic often showing mitotic figures The nuclei were smaller in diffuse carcinomas smooth-edged more regular in form evenly or only faintly hyperchromatic often however pyknotic Mitotic figures were generally fairly difficult to establish

The cells of intestinal type carcinoma which lined the glandular lumina were as a rule fairly well polarized columnar cells (Fig 3) The apical surface of the cells continued unbroken from one cell to another It was possible to demonstrate a well developed brush border


1
2

in 83 per cent (146 176 cases) of all intestinal type carcinomas and in 9? per cent (146 159) of the intestinal type carcinomas forming glandular structure The cells lining the lumina which occasionally were encountered in diffuse carcinomas were unpolarized randomly grouped and the apical surface from cell to cell was of a wavy form (Fig 5) Surface differentiation of brush border type was demonstrated in 11 per cent (10 94) of all and in 48 per cent (10 21) of diffuse carcinomas forming glandular structure The formation however was generally more uneven and the bristles were more sparse than in the brush border seen in the cells of intestinal type carcinomas

Secretion Eighteen per cent (30/171) of the intestinal type carcinomas showed no cells containing secretory products In diffuse carcinomas all the tumours studied by mucus staining methods displayed secreting cells Most of the secreting intestinal type carcinomas (61 per cent 86 141) had only few secreting cells among the protoplasmatic cells (Fig 6) Ninety per cent (79 88) of the diffuse carcinomas displayed secretion over extensive areas in nearly all of the tumour cells (Fig 7) In the intestinal type carcinomas secretion almost always formed a distinctly defined theca in the cytoplasm in diffuse carcinomas it appeared always to be evenly distributed in the cytoplasm

The extracellular secreted mucus was located chiefly in the glandular lumina in intestinal type carcinomas and dispersed in the stroma in diffuse carcinomas Extracellular mucus was missing completely in 23 per cent (39 171) of the intestinal type and in 63 per cent (59 94) of the diffuse carcinomas

Colloid carcinoma arose from carcinomas of intestinal as well as of diffuse type This was seen in 11 per cent (19 171) of the intestinal type and in 21 per cent (20 94) of the diffuse carcinomas The structure of the colloid carcinoma differed in these two carcinoma types Colloid carcinoma of intestinal type mostly arose secondarily from adenocarcinoma when the glandular lumina were dilated and split by profuse mucus (Fig 8) It originated more rarely from the transformation of the cells of a solid carcinoma into mucus filled signet ring cells (primary colloid carcinoma of the colon *Raiford* 1932) Colloid carcinoma of diffuse type arose from mucus masses which accumulated around isolated cells or loose cell clusters (Fig 9) In some part of the tumour

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Fig 3 Glands of intestinal type carcinoma The cells are fairly well polarized high

Fig 4

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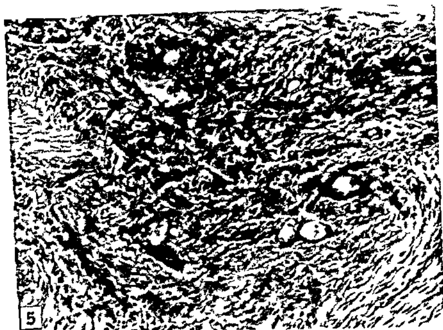


Fig 5

The miniature glandular lumina sometimes occurring in diffuse carcinomas The cells are not polarized and correspond structurally the other cells of diffuse carcinoma Haematoxylin mucicarmine staining × 160

these colloid carcinomas regularly displayed the original structure of the diffuse carcinoma

The histochemical properties of mucus did not differ distinctly in the intestinal type and diffuse carcinomas The secretion stained as pure acid mucus in 44 per cent (58/132) of the intestinal type carcinomas Slightly more frequently (in 52 per cent, 69/132) the secretion stained in addition to acid stains with Best s carmine which demonstrates neutral mucus Cases in which the mucus of intestinal type carcinoma stained in the manner of purely neutral mucus were few (4 per cent 5/132) In diffuse carcinomas, two kinds of secreting cells often occurred in the same tumour It is possible that they represented two different secretory phases of the same cell type One cell type contained more profuse the other more scanty mucus The mucus of the former usually stained like acid mucus or, both reactions might be positive The secretion of the latter almost always stained like neutral mucus Thus 88 per cent (81/92) of the diffuse carcinomas contained secreta which stained in the manner of acid as well as of neutral mucus The secreta stained purely like neutral mucus in 10 per cent (9/92) and purely like acid mucus in 2 per cent (2/92) of the cases

nterochromaffin cells, both argyrophil and argentaffin ountered in both carcinoma types However, tumours



Fig 6 7

Fig 6 Secretion of mucus in intestinal type carcinoma Only a few secreting cells are brought out by PAS The mucus forms a distinctly defined theca in the middle of the cell The glandular lumina contain a greater quantity of secreted mucus PAS technique × 160

Fig 7 Secretion of mucus in diffuse carcinoma The majority of the tumour cells contain mucus It is evenly distributed in the cytoplasm and therefore seems to be indistinctly delimited PAS technique × 160

with enterochromaffin cells were considerably more frequent and the cells were more profuse in diffuse than in intestinal type carcinomas

Mode of growth Intestinal type carcinoma usually spread to a clearly and often distinctly defined area The type of tumour tissue varied in the different parts of the carcinoma a tumour forming large glandular lumina in the centre could be scirrhous at the margin of its spread frequently forming only small solid cell cords The original intestinal type cell structure was distinctly visible even in such peripheral areas Diffuse carcinoma did not form as well defined tumours The mode of growth of the tumour tissue at the margin of the spread did not differ from modes of growth in the other parts of the tumour

Generally the intestinal type carcinoma did not spread more widely in the mucosa than above the infiltrating part of the tumour It tended to ulcerate over the entire area in which it reached the mucosa Diffuse carcinoma was characterized by a wider spread inside the mucosa (contiguous plaque phenomenon *Collins & Gall* 1952) The infiltrated mucosa was often preserved from ulceration because of the superficial parts of normal mucosa that were revealed

Connective tissue proliferation varied quantitatively in both types of *carcinoma* However the almost medullary mode of growth encountered fairly frequently in the intestinal type carcinomas was rare in diffuse carcinomas Scirrhous structure was more typical of diffuse *carcinomas but* was not rare in carcinomas of intestinal type Scirrhous *structure originated* occasionally in diffuse carcinomas also without stromal proliferation the tumour cells spreading especially in the tunica muscularis and serosa in fairly *sparse clusters*

The inflammatory *cell infiltration* pertaining to host reaction and independent of ulcerations or necroses was usually profuse in intestinal type *carcinomas* it could be *very pronounced* and if so was formed largely of polymorphonuclear leucocytes *In several* cases the inflammatory cell infiltration was concentrated at the margin of spread of the tumour It was mostly *more faint* sometimes very slight in diffuse carcinomas the inflammatory cells were usually *mononuclear*

CORRELATIONS BETWEEN INTESTINAL TYPE AND DIFFUSE GASTRIC CARCINOMA AND OTHER CHARACTERISTICS OF THE DISEASE

Sex The great majority 65 per cent (465 patients) of the cases with intestinal type carcinoma were men 35 per cent (250) women The preponderance of men was distinctly less marked among patients with diffuse carcinoma Men 54 *per cent (240)* women 46 per cent (201)

Age The mean age of the patients with intestinal type carcinoma was 55 4 years (calculated by decade groups), the age of patients with diffuse carcinoma being clearly lower, 47 7 years The age distribution was distinctly different in both groups (*Table 1a*) Thirteen per cent



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F g 6 Secretion of mucus in intestinal type carc noma Only a few secreting cells are brought out by PAS The mucus forms a distinctly defined theca in the middle of the cell The glandular lumina contain a greater quantity of secreted mucus PAS technique × 160

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of the patients with intestinal type carcinoma were below the age of 50, the peak incidence was seen in the age group 60 69 years, 55 6 per cent of the patients were above 60 A distinctly greater proportion, 37 1 per cent of the patients with diffuse carcinoma were below the age of 50 the peak incidence being seen in the age group 50 59 years and 30 5 per cent of the patients were above the age of 60 The proportion of diffuse carcinomas in all gastric carcinomas was 70 per cent in the age group below 40 years but only 22 per cent in the age group above 60 years (Table 1b)

TABLE 1a

Age Distribution of Patients with Intestinal Type and Diffuse Gastric Carcinoma

		Total	10–	20	30–	40	50	60–	0–	80–
Intestinal type	cases	701		–	17	74	220	282	106	2
carcinoma	%	100			2 4	10 6	31 4	40 2	15 1	0 3
Diffuse	cases	429	2	5	50	102	139	96	35	–
carcinoma	%	100	0 5	1 2	11 6	23 8	32 4	22 4	8 1	

TABLE 1b

Proportion of Diffuse Gastric Carcinomas in all Carcinomas in the Different Age Groups

		Total	Below 40 yrs	40-49 yrs	50-59 yrs	Above 60 yrs
All carcinomas	cases	1312	82	196	425	609
Diffuse ca	cases	429	57	102	139	131
	%		70	52	33	22

Changes in the normal mucosa close to the tumour (Table 2a) The gastric mucosa in the immediate vicinity of intestinal-type carcinomas was more rarely of a normal or near normal structure than in diffuse carcinomas (10 and 23 per cent) Marked signs of chronic atrophic, atrophic hyperplastic, or hyperplastic gastritis in the surrounding mucosa were encountered in 88 per cent of the intestinal type and in 45

Fig 8–9

Fig 8 Colloid carcinoma developing from an intestinal type carcinoma The epithelium of the glandular lumina of the adenocarcinoma also seen in the picture becomes more shallow on account of the profuse secretion and breaks loose pieces of the columnar epithelium ... masses Haematox...

Fig 9 Colloi... picture ... mucus ... the ma... form pi... ... but they do not ... epithelium Haematoxylin van Gieson staining × 100

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diffuse carcinomas (26 per cent) were reported to be excavating tumours Intestinal-type carcinoma formed more rarely an infiltrate of the linitis plastica type (15 per cent) than diffuse carcinomas (43 per cent)

The localization of the tumour in the stomach displayed no definite correlation with the histological type Fifty five per cent (277/498) of the intestinal type carcinomas and 60 per cent (183/306) of the diffuse carcinomas were seen in the pylorus and antrum, 31 (152) and 29 per cent (88), respectively, were at sites higher up in the lesser curvature and in the body, and 13 (62) and 4 per cent (13) in the cardia One per cent (7) of the intestinal type and 7 per cent (22) of the diffuse carcinomas had spread throughout the stomach wall

Prognosis To assess the prognosis, a material of 153 patients was available to whom curative treatment was given (*Lauren et al* 1962) In this series intestinal type carcinoma had most favourable prognosis The postoperative survival rate was below 1 year in 22 per cent of the cases of intestinal type and 32 per cent in cases of diffuse carcinoma, while 36 and 32 per cent of the patients, respectively, lived for 1–3 years and 43 and 35 per cent for more than 3 years

DISCUSSION

While adenocarcinomas of intestinal-type have been rather easy to classify histologically, diffuse carcinomas cause more problems and opinions differ as to the nature of these tumours The terminology applied to diffuse carcinomas is diversified and the types of tumour included in the various, arbitrarily formed groups are different *Krompecher* (1910) regarded some of these tumours as inflammatory changes and employed the old name linitis plastica which is suggestive of inflammation The term most commonly used has been scirrhous carcinoma (*e g Verse* 1908 and *Borrmann* 1926) Usually this group, however, has included adenocarcinomas containing profuse stroma *Borrmann* also described diffuse carcinoma as a separate type, which is in closer agreement with the present author's specification of the diffuse carcinoma type Other descriptive classifications corresponding more or less herewith are solid diffuse carcinoma (*Tuomikoski* 1937), fibrous carcinoma (*Konjetzny* 1938), muciparous scirrhus (*Eker & Efskind* 1952), disseminated carcinoma (*v Albertini* 1955), diffuse scirrhous carcinoma (*Evans* 1956) and undifferentiated carcinoma (*Takagi & Sama* [illegible]

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(195[illegible] separate type of tumour and draws the attention to the unusual mode of mucus secretion and the presence of

per cent of the diffuse carcinomas The mucosa around diffuse carcinoma fairly often (32 per cent) displayed high fold formation which was covered by gastric surface epithelium and differed from that occurring in chronic gastritis Similar fold formation was encountered only exceptionally in carcinomas of intestinal type

TABLE 2a

Changes in the Mucosa Surrounding the Carcinoma in Intestinal Type and Diffuse Gastric Carcinomas

		Total	Nearly normal	Chronic gastritis	Large superficial epithelial folds
Intestinal type carcinoma	cases	329	34	288	7
	%	100	10	88	2
Diffuse carcinoma	cases	271	62	123	86
	%	100	23	45	32

TABLE 2b

Occurrence of Intestinal Metaplasia in the Mucosa Surrounding the Carcinoma in Intestinal Type and Diffuse Gastric Carcinomas

		Total	Absent	Scanty	Fairly profuse	Profuse
Intestinal type carcinoma	cases	536	48	132	157	199
	%	100	9	25	29	37
Diffuse carcinoma	cases	324	145	101	54	24
	%	100	45	31	17	7

Intestinal metaplasia in the surrounding mucosa was distinctly more frequent and widespread in intestinal-type than in diffuse carcinomas (Table 2b) Metaplasia in the normal mucosa included in the specimen was absent in 9 per cent of the intestinal-type and in 45 per cent of the diffuse carcinomas On the other hand, profuse or fairly profuse metaplasia was present in 66 per cent of the intestinal-type and in 24 per cent of the diffuse carcinomas The difference in the incidence of intestinal metaplasias in intestinal type and diffuse carcinomas emerged in all age groups In the age group below 50 years, cases without metaplasia in the mucosa totalled 15 per cent (9/62) in intestinal type carcinoma, 58 per cent (77/132) in diffuse carcinomas, in patients aged 50–59 the percentages were 12 (19/156) and 39 (44/112), respectively, in patients above 60-years it was 7 (18/253) and 20 (20/99) per cent

The macroscopic features of the tumour showed some correlation with the histological main types Of the intestinal type carcinomas, 60 per cent (215/361) were described as polypous or fungating, the corresponding percentage in diffuse carcinomas was 31 (73/235) An equally great proportion both of intestinal-type (25 per cent) and

intestinal type carcinoma For this comparison the material obtained in an earlier investigation was used (*Lauren* 1961) Although all the sub types of intestinal type gastric carcinoma occur in *carcinomas of the colon* as well, the incidence of the various sub types in the intestines *differs* from the incidence in the stomach The most significant difference is the more profuse occurrence of papillary structures in carcinomas of the colon It is possible that the difference is due to the different mode of origin Carcinomas of *the colon obviously* arise mostly from adenopapillomas, gastric carcinomas, in contrast, from a changed but not polypous mucosa The diffuse type is fairly rare among carcinomas of the colon The present author has encountered only two diffuse carcinomas assumed to be primary in the intestines *One of these was found in a girl aged 13* in the flexura lienalis of the colon, the other derived from the rectum It was not possible in any of these cases to rule out the possibility of a concomitant gastric carcinoma The rarity of diffuse colonic carcinoma has also been reported in the literature (*Laufman & Saphir* 1951, *Fahl et al* 1960), together with the fact that when these carcinomas actually occur in the intestines they are metastases of gastric carcinoma (*Dixon & Stevens* 1936)

As *diffuse* carcinomas have no glandular structure they are always

... that the differentiation of their cells has progressed far Secretion of *mucus is more common as* well *as more profuse in these* tumours than in intestinal type carcinomas Enterochromaffin cells are encountered more often The characteristics of malignant tumours, cellular atypia and numerous mitotic figures are more prominent in intestinal type *than in diffuse* carcinomas The evaluation of malignancy on the basis of the microscopic structure has not been as satisfactory in cases of gastric carcinomas as in the grading of other carcinomas (*Schindler et al* 1941, *Steiner et al* 1948, *Hoerr* 1954, *Lewin* 1960) *However, it is known that the prognosis* is poorer in diffuse than in intestinal type carcinomas, which is also born out in the present material The reason hereof may be some ...

... extensive area They do not form the distinct margin of spread which is often typical of intestinal type carcinomas and the existence of which is important for a favourable prognosis (*Murakami et al* 1959 *Eker & Efskind* 1960) Another factor in the poor prognosis is that diagnostic symptoms in these tumours ...

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enterochromaffin cells Mucous cell carcinoma was by *Mulligan & Rember* (1954) considered to be a histogenetically specific tumour with its own biologic behaviour *Nagayo & Komagoe* (1961) stated in their study on mucosal carcinomas that diffuse carcinomas which metastasize as single cells constitute a basic type of their own which does not change into an adenocarcinoma forming definite glands

The specific difference between intestinal-type and diffuse carcinoma revealed by structural study is corroborated by comparative observations of the structure of the primary tumour and the metastases The present material included 202 cases with lymph node metastasis As a rule the structure of the tumour tissue in the lymph node would correspond to the main type of primary tumour Usually structure of intestinal-type carcinoma would be differentiated equally far in the metastasis and in the primary tumour The cells of diffuse carcinomas were often larger in lymph node metastases than in the primary tumour During proliferation in the sinuses the cells would often form dense masses and thus the tumour tissue might be more solid than tissue in the primary tumour Several other investigators have also reported that the basic type of gastric carcinoma remains the same when the tumour spreads, but that variations occur because of topical conditions at the growth site (*Bohmig* 1937, *Oota & Tanaka* 1951b, 1952, *Tauchi & Sato* 1958, *Tauchi et al* 1960)

The histological structure of a gastric carcinoma can remain unchanged for a longer period which feature is evident from the 13 cases in the series in which the tumour recurred more than one year after the first operation The structure also of the new tumour, was usually of the same sub type as that of the primary tumour As emphasized by *Willis* (1960), the occurrence of true progression by which the structure of a tumour is changed is a rare phenomenon

Oota & Tanaka (1951a) turned their attention to the difference of the type of inflammatory cell infiltration in intestinal-type and in diffuse carcinoma which feature has been established in the present study They attributed the marked inflammatory cell infiltration seen in adenocarcinomas to infection of the secretion retained in the glandular lumina An argument against this theory is the finding that colloid carcinomas of diffuse type usually display only scanty inflammatory cell infiltration In the opinion of the present author, a more probable explanation of inflammatory cell infiltration unrelated to ulceration and necroses is the reaction of the organism to carcinoma This hypothesis was advanced *e g* by *Black et al* (1954) and *Larmi & Saxen* (1963) This part of the host reaction seems to be of a different nature in intestinal-type and in diffuse carcinoma The other component of the host reaction, the proliferation of connective tissue, is also different in the two types of gastric carcinoma

Comparison of the structure of the tumour with that of carcinomas of the colon was one of the criteria used for the definition of the

and intestinal type carcinomas is easier and more reliable than the systems used in earlier methods This classification may serve to improve the compatibility of different gastric carcinoma studies

The principal significance of differentiation between intestinal type and diffuse gastric carcinoma is however that the tumours of these two types differ not only structurally but also in their other characteristics Such difference is not allowed for in the purely descriptive histological types of gastric carcinoma in common use The observations made here motivate the assumption that intestinal type and diffuse carcinoma might have an at least somewhat differing aetiology and pathogenesis

SUMMARY

Two histological main types could be distinguished in gastric carcinomas by a structural and histochemical study of a surgical material comprising 1 344 cases Intestinal type carcinoma accounted for 53 per cent and diffuse carcinoma for 33 per cent of all the gastric carcinomas The carcinomas of these main types had their own typical features of general structure and cell structure secretion of mucus and mode of growth The proportion of men and older patients was greater in the intestinal type group than in the group of diffuse carcinoma Gastritic changes in the surrounding mucosa and the incidence and extent of intestinal metaplasias were greater in intestinal type carcinomas The prognosis was poorer in diffuse than in intestinal type carcinomas

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appraisal of the histological malignancy in intestinal-type and in diffuse carcinomas

The high incidence of diffuse type tumours characteristic of gastric carcinomas in the young age groups has also been established by several other investigators (*Oota & Misu* 1959, *Lewin* 1960, *Tamura & Curtiss* 1960, *Nagayo & Komagoe* 1961) *Jarvi & Lauren* (1951), *Sato et al* (1959) and *Tauchi et al* (1960) found, correspondingly, that carcinomas in older patients mostly are adenocarcinomas The relatively high incidence of diffuse carcinoma in women has also been noted by *Lewin* (1960) and *Tamura & Curtiss* (1960)

Opinions on the pathogenesis of gastric carcinoma have been highly conflicting on the point whether or not carcinoma is preceded by chronic gastritis with intestinal metaplasia Especially the workers (*e g Tuomikoski* 1936, *Siurala & Seppala* 1960) who were familiar with *Saltzman*'s (1913) and *Konjetzny*'s (1928, 1938) studies, regard chronic gastritis as pathogenetically important But its importance is denied by several Anglo-Saxon authors (*e g Guiss & Stewart* 1943, *Magnus* 1946, *Hebbel* 1949) *Schindler* (1940) and *Morson* (1956) postulate that a part of carcinomas are preceded by gastritis The present authors's observations suggest that the precancerous change in the mucosa is different in intestinal-type and in diffuse carcinomas Chronic gastritis and intestinal metaplasias in the mucosa around the tumour tissue are often lacking in diffuse carcinomas, as *Nagayo & Komagoe* (1961) also have seen in their study on superficial carcinomas In intestinal-type carcinoma, on the other hand, the tumour almost always borders on the mucosa which shows chronic gastritis and metaplasia On the other hand, similar high fold formation covered by surface epithelium of the gastric mucosa to that seen on the margins of peptic ulcers are often seen on the boundaries of diffuse carcinoma *Oota & Misu* (1959) reported that 59 3 per cent of the diffuse carcinomas in their series originated in peptic ulcer It was not possible in the present series to examine sufficiently thoroughly the correlation between ulcer and carcinoma However, the precancerous, morphologically demonstrable lesion seems to be fairly limited regionally in diffuse carcinoma, whereas intestinal-type carcinoma is preceded by chronic gastritis in an extensive area of the mucosa The predominance of women among patients with diffuse carcinoma and the young age of these are probably also indicative of the different character of the lesion preceding this carcinoma type as compared with the lesion preceding intestinal type carcinoma (*Jarvi & Lauren* 1951, p 39, Table 8)

The present study shows that intestinal type and diffuse gastric carcinoma, which include the large majority of the gastric carcinomas, constitute two histological basic types which are individually different There is no requirement for a more detailed descriptive classification in examination and in routine diagnostic The classification into diffuse

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could be clearly demonstrated where grouped to ductogenic infiltrating carcinomas with adenomatous structure

RESULTS

The material and the results are shown in Table 1 The following classification of the stage of the tumour at operation was used

Stage I	The neoplasm was confined to the breast tissue
Stage II	The axillary lymph nodes were involved
Stage II	The axillary lymph nodes were involved
Stage IV	Distant metastases were present

The majority, thirteen cases, belonged to groups III or IV No correlations between the stage of tumour, the histological type of tumour and the sex chromatin frequency could be seen

TABLE 1
The Data on the Investigated Mammary Cancer Patients

Patient	Age	Stage of tumor at operation	Histologic type of carcinoma	The incidence of chromatin bodies per cent	Presence of more than one body
1	62	II	infiltrating carcinoma with fibrosis	0.8	—
2	70	I	infiltrating ductogenic carcinoma	1.3	—
3	58	II	infiltrating lobular carcinoma	3.0	—
4	60	III	infiltrating papillary carcinoma	2.8	+
5	72	II	infiltrating lobular carcinoma	9.5	+
6	56	III	infiltrating carcinoma with fibrosis	4.3	+
7	63	III	infiltrating ductogenic carcinoma (adenocarcinoma)	10.0	+
8	54	III	infiltrating comedocarcinoma	4.1	—
9	80	IV	infiltrating ductogenic carcinoma	0.6	—
10	60	II	infiltrating ductogenic carcinoma	2.0	+
11	62	III	infiltrating carcinoma with fibrosis	1.1	—
12	62	III	infiltrating lobular carcinoma	0.6	—
13	63	I	infiltrating carcinoma with fibrosis	11.6	+
14	54	III	infiltrating ductogenic carcinoma (adenocarcinoma)	0.5	+
15	55	III	infiltrating ductogenic carcinoma	0.6	—
16	80	III	infiltrating ductogenic carcinoma	4.5	—
17	49	III	infiltrating ductogenic carcinoma	1 01	—
18	50	III	medullary carcinoma	3.0	+
19	76	II	infiltrating ductogenic carcinoma	0.3	—
20	62	III	infiltrating ductogenic carcinoma	1.2	—
21	74	I	infiltrating ductogenic carcinoma	6.3	+

Acta path et microbiol scandinav 64 50–54 1965

The Department of Pathological Anatomy (Head Prof Osmo Järvi, M D) and The Department of Surgery (Head Prof Sauli Viikari M D), University of Turku Turku Finland

A CYTOLOGICAL STUDY ON THE SEX CHROMATIN IN CARCINOMA MAMMAE

By

ILMARI LINDGREN[1] and EERO ELOMAA

Received 9 xi 64

The sex chromatin, also called the Barr body, is a condensed mass of chromatin which is seen in interphase female nuclei This body is derived from two X chromosomes (*e g Barr & Carr* 1962) The sex chromatin can be seen even in vivo (*Schwarzacher* 1963) A chromosomal origin of the sex chromatin is also compatible with the observed increase in the number of sex chromatin in direct proportion to the degree of polyploidy (*Klinger & Schwarzacher* 1960, *Mittwoch et al* 1963) For example, cells containing 49 chromosomes with a sex chromosome constitution XXXXY may show two or three Barr bodies (*Atkins et al* 1963)

It was hoped to determine the nuclear sex in hormone related tumours In the case of mammary cancer, it has been claimed that the "male" tumours should be more sensitive to oestrogens, and that on the contrary androgens should have greater effect on "female" neoplasms (*Kimel* 1957, *Blumel et al* 1963, *Regele et al* 1964) The evidence is lacking of a direct relation of hormones to nuclear sex (*Pfeifer* 1962, *Platt et al* 1964) and especially the nuclear sexing of the malignant growth still seems to be questionable (*Tavares* 1962) We have therefore studied the problem in mammary carcinomas using the original technique for sex chromatin, which was applied on cut tumour tissue

MATERIAL AND METHODS

21 mammary carcinoma patients of female sex were operated and the resected tumour was immediately taken to the Pathology Laboratory where the diagnosis was made by freeze section examination From the unfrozen part of the tumour a smear preparation was made Usually one of the prepared four slides contained enough cells but in some scirrhous carcinomas it was useful to make more slides to get enough cells for examination

The smear preparations were stained thionin after previous hydrolysis with 5 N HCl according to the method of *Klinger & Ludwig* (1957) All the microscopic examinations were done with oil immersion objective (100 ×) and 600 well preserved cells were examined The number of sex chromatin bodies was counted in per cent Only those bodies which lay directly against the inner surface of the nuclear mem

1 Junior Research Fellow of the Finnish State Medical Council

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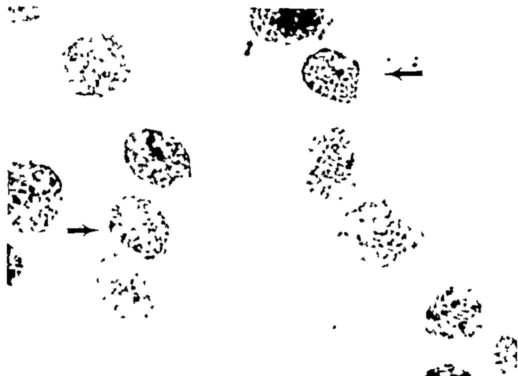


Fig 1

A smear preparation from a cut surface of a carcinoma mammae This slide derives from patient number 18 Several cancer nuclei showing condensed chromatin masses both centrally and marginally The arrows indicate two nuclei having duplicate or tripilacate marginal bodies Thionin after acid hydrolysis Reproduced at 1500 ×

The mean frequency of such bodies which lay directly against the inner surface of the nuclear membrane was in this material 3 3 per cent This showed a range between 0 3 and 11 6 per cent The proportion of sex chromatin-positive cells was variable from one field of vision to another even in the same specimen Even the size of these bodies showed a variance from the size of the normal sex chromatin body down to the minutest ones detectable However, only clearly visible ones having a size approximately half of the normal sex chromatin or greater weer counted In nine cases of the whole material one could detect nuclei which showed two or three bodies lying against the inner surface of the nuclear membrane These cases which showed the multiplication of the chromatin body also showed the highest incidence of the bodies

Fig 1 shows a typical smear preparation from a cut tumour surface The nuclei are solitary and the chromatin masses can easily be seen in both central or marginal location

DISCUSSION

The results obtained in this study differ quantitatively from those of certain workers who have published reports on the same theme

(*Kimel* 1957, *Blumel et al* 1963, *Regele et al* 1964) They had sometimes seen the sex chromatin in 40 per cent or more in the nuclei of mammary cancer cells (*Moore & Barr* 1957) It should be pointed out that in these studies a different technique was used At first the examinations were done on paraffin sections of varying thickness However, the quantitative determination of sex chromatin from cut sections does not seem to be very reliable Secondly, in some studies (Kimel 1957, *Regele et al* 1964) sections were stained with haematoxylin without any pretreatment By this method the nucleolus is also stained and this may give a source of error Apparently the more reliable method to determine the incidence of chromatin bodies of the nuclei is to study smear preparations which contain isolated cells The staining method should only reveal the nuclei acids of desoxyribose type

The main chromosomal feature of cancer cells is polyploidy with a wide variation of chromosome number, and even abnormal chromosomes can be found (*Makino et al* 1959) In mammary carcinomas the chromosome number has been shown to vary widely between 65 and 210 (*Spriggs et al* 1962) The neoplasm arising in female hosts are derived from cells which originally contain two X chromosomes During numerous irregular mitoses this constitution is probably often lost For example in tripolar or multipolar mitoses it is impossible that all the daughter nuclei were to contain two X chromosomes Therefore it would be unexpected if two X chromosomes were able to pass through numerous pathological mitotic cycles in certain types of cancer It is even possible that in a cancer cell which may contain two X chromosomes *these are not able* to condense into a sex chromatin body Hence if a [illegible]

b [illegible]

a [illegible] the other a male carcinoma

It is evident that some cancers in their nuclei have a [illegible] hroma- [illegible] may be [illegible] 1960) [illegible] may vary in size, and its frequency is generally low and shows a low sliding scale from one tumour to another Thus, no exact determination of the sex of a given tumour can be made On the other hand it is not sure that *this body in cancer nuclei* is formed of two X chromosomes at all This point of view would need further investigation with tritium labelled thymidine by which the X chromosomes could be demonstrated

SUMMARY

In twenty one mammary carcinomas from female hosts the fresh tumour was sectioned, and a smear preparation was made from the cut surface and stained by a method commonly accepted in sex chro-

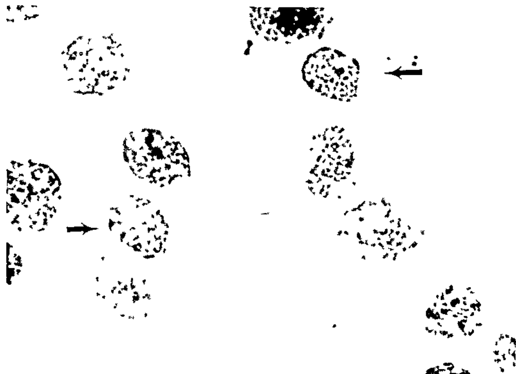


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It is evident that some cancers in their nuclei have a distinct chromatin body near the nuclear membrane and even multiple bodies may be present as shown here and by others (*Barr & Carr* 1960 *Atkin* 1960) This body may vary in size and its frequency is generally low and shows a low sliding scale from one tumour to another Thus no exact determination of the sex of a given tumour can be made On the other hand it is not sure that this body in cancer nuclei is formed of two X chromosomes at all This point of view would need further investigation with tritium labelled thymidine by which the X chromosomes could be demonstrated

SUMMARY

In twenty one mammary carcinomas from female hosts the fresh tumour was sectioned and a smear preparation was made from the cut surface and stained by a method commonly accepted in sex chro

matin studies The condensed chromatin masses lying directly against the nuclear membranes were counted The mean frequency of these bodies was 3 3 per cent (range from 0 3 to 11 6 per cent) In nine tumours duplications or triplications of the body were observed the size of the body also varied

It was concluded that exact determination of sex of mammary carcinoma was difficult and maybe impossible

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STARCH GRANULOMAS AS A PROBLEM IN SURGICAL PATHOLOGY

By

L SAXÉN and E SAXÉN

Received 3 x 64

Both clinical experience and experimental observations have shown that the talc powder earlier used in surgical gloves has an irritating effect (*Owen* 1936, *Lichtman et al* 1946, *Saxen & Tuovinen* 1947, *Seeling* 1948) Consequently, a number of attempts have been made to replace it by other, less irritative materials, suggestions have included potassium bitartrate *(Seeling et al* 1943), liquid lubricants (*Winster et al* 1962) and star h
sium oxide
1948, *Gra*
studies with starch powders gave promising results, and indicated that the material was completely absorbed within the course of a few weeks (*Postlethwait et al* 1949a, 1949b) Nevertheless subsequent experiments resulted in contradictory findings which indicated that this material also exerted irritative effects when brought into contact with tissues (*Lee et al* 1952) Many recent studies have confirmed these findings under experimental conditions (*Ising* 1960, *Myers et al* 1960, *Hupe* 1962, *Zullig* 1962) Moreover, a number of authors have reported
cases
ave caused reac-
foreign material
, *Myers et al* 1960, *Ising* 1960, *Walczak & Collura* 1962, *Zullig* 1962, *E Saxen & Munalainen* 1963) Nevertheless, no conclusive evidence has been presented to show whether it is the starch itself which is the irritative compound, or whether some other components of the powder are responsible for this

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the lesion is not sufficiently well-known among pathologists, have led us to the making of this report In what

TABLE

ase	Age	Sex	Primary disease		Post-operative
			Diagnosis	Operation	Time of appearance
1	34	♀	Carcinoma of colon	Hemicolectomy	4 months
2	13	♀	Cystadenoma of ovarium	Ovariectomy	1 month
3	17	♀	Appendicitis Salpingitis	Appendectomy Salpingectomy	2 months
4	49	♀	Cholecystitis	Cholecystectomy	1 month
5	22	♀	Hematoma retroperitoneale	Laparatomy	2 weeks
6	34	♀	Carcinoma of stomach	Gastric resection	5 weeks
7	67	♂	Carcinoma of stomach	Gastric resection	4 months
8	37	♀	Carcinoma of cecum	Hemicolectomy	1 month
9	43	♂	Carcinoma of rectum	Abdomino perineal resection	6 months
10	56	♂	Hodgkins disease	Lymph node excision	1 month
11	64	♂	Basal cell carcinoma of the skin	Excision of the tumor	3 weeks
12	72	♂	Actinomycosis of the skin	Skin biopsy	3 months
13	4	♂	Ruptured liver?	Explorative laparatomy	2 months
14	28	♀	None	None	– –

follows, cases are presented from daily routine and attention is focused on the typical microscopical changes, the histological diagnosis of these lesions, and especially on the limitations and the errors which may arise in such a diagnosis

CLINICAL DATA

In the light of our own cases, and earlier reports, starch reactions can be divided into three categories in accordance with their clinical course, a starch peritonitis, recurrence and a persistent local inflammation Some data concerned with these three groups, together with some typical examples, are given below The material is presented in Table 1

Starch peritonitis

As a rule, the symptoms make an appearance 1 to 3 months after a laparatomy, the most typical of them being abdominal pains resembling

1

symptoms	Pre-operative surgical diagnosis	Reoperation	
Symptoms		Operation	post-operative pathologic diagnosis
Acute obstruction	Obstruction Carcinomatosis	Laparatomy	Starch granuloma
Abdominal pains	Peritonitis Carcinomatosis	Laparatomy	Starch granuloma
Acute abdominal pain	Adhesions	Laparatomy	Starch granuloma
Abdominal pains	Peritonitis Adhesions	Laparatomy	Starch granuloma
Abdominal pains	Peritonitis	Laparatomy	Starch granuloma
Obstruction	Carcinomatosis	Laparatomy	Starch granuloma
Obstruction	Carcinomatosis	Laparatomy	Starch granuloma Metastatic carcinoma
Tumor in scar	Metastasis	Excision	Starch granuloma
Tumor in scar	Metastasis	Excision	Starch granuloma Metastatic carcinoma
Recurrence in scar	Recurrence	Excision	Starch granuloma
Recurrence in scar	Keloid	Excision	Starch granuloma
Recurrent inflammation	Chronic inflammation	Excision	Starch granuloma
Tumor in epididymis	Tumor epididymis	Epididymectomy	Starch granuloma
Peritonitis acute obstruction	Peritonitis obstruction	Laparatomy	Starch granuloma

those due to adhesions, and symptoms of acute occlusion A relaparatomy reveals a rather generalized peritoneal spread of small wh...

below ... in our laboratory (1 to 7), and one of them is presented

Case 2 A 13-year-old girl was subjected to operation following a history of abdominal pains over a period of one year The clinical diagnos... was Cysta ovar... I dx) being... return... minal ... weeks later the abdo-

... are numerous cornsized nodules, of

which some were sent to the pathologist with the diagnosis "Carcinosis peritonei" Microscopical examination disclosed a typical starch granuloma with no malignant cells

It may be added that the patient remembered suffering from acute eczema as a child after the consumption of eggs and strawberries After the operation, there was noted a strong reaction to the plaster, accompanied by eosinophilia Later, a skin-test was made with BIO-SORB starch powder, however this yielded a negative result

Recurrence

This was the clinical diagnosis in four of the cases (No 8 to 11) In two cases, the local tumour was observed in a laparatomy scar, in the third at the site of an earlier biopsy for lymphogranulomatosis, and in the fourth case a keloid-like tumour was found in the wound after removal of a basal-cell carcinoma Here, as in the preceding group, the interval from the previous operations was usually short, varying from 3 weeks to 6 months The following example will illustrate a typical history

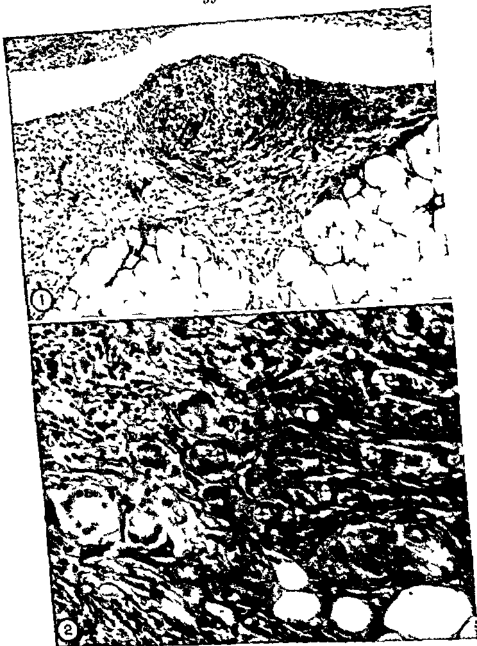
Case 10 An enlargement of the cervical lymph nodes led to a biopsy in a 56-year-old male The diagnosis of the pathologists was malignant lymphogranumatosis, subsequently "Sendoxan"-treatment was initiated Four weeks later, a tumour was still present at the site of the previous biopsy, and a new sample was taken for microscopical examination No lymph gland tissue was seen in the sample, which consisted of granulation tissue showing typical foreign-body reaction with abundant starch-granules

Persistent inflammation

In some cases reported by *Ising* (1960), the clinical symptoms of the starch granulomas consisted of persistent inflammation Subsequent to a Luc-Caldwell operation, the symptoms of chronic sinusitis persisted, and histological examinations made in conjunction with a reoperation demonstrated a typical foreign-body reaction with starch-granules in the mucous membranes Case No 12 in our series belongs to this clinical group biopsy of a cutaneous lesion (Actinomycosis) was followed by local symptoms that were clinically indicative of a persistent infection The area was excised three months later, and histological examination revealed a typical starch-granuloma

Exceptional spreading of starch powder

A rather exceptional spreading of starch-containing preparations has been demonstrated in two of our cases (Cases 13 and 14) It is only na-



Figs 1 2

Fig 1 ... Two ... ight

Fig 2 ... d in ... eign ... eson

Magnification × 350

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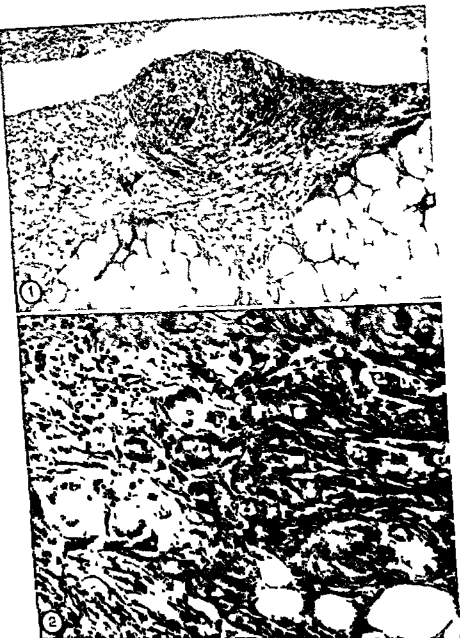
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In some cases reported by *Ising* (1960), the clinical symptoms of the starch granulomas consisted of persistent inflammation Subsequent to a Luc-Caldwell operation, the symptoms of chronic sinusitis persisted, and histological examinations made in conjunction with a reoperation demonstrated a typical foreign-body reaction with starch-granules in the mucous membranes Case No 12 in our series belongs to this clinical group biopsy of a cutaneous lesion (Actinomycosis) was followed by local symptoms that were clinically indicative of a persistent *infection* The area was excised three months later, and histological examination revealed a typical starch-granuloma

Exceptional spreading of starch powder

A rather exceptional spreading of starch containing preparations has been demonstrated in two of our cases (Cases 13 and 14) It is only na-



F gs 1 2

F g 1 A low power v ew of a fore gn body granuloma in the peritoneal lining Two starch like granules are seen in the picture taken in half polarized light

Magn ficat on × 350

which some were sent to the pathologist with the diagnosis "Carcinosis peritonei" Microscopical examination disclosed a typical starch granuloma with no malignant cells

It may be added that the patient remembered suffering from acute eczema as a child after the consumption of eggs and strawberries After the operation, there was noted a strong reaction to the plaster, accompanied by eosinophilia Later, a skin-test was made with BIO SORB starch powder, however this yielded a negative result

Recurrence

This was the clinical diagnosis in four of the cases (No 8 to 11) In two cases, the local tumour was observed in a laparatomy scar, in the third at the site of an earlier biopsy for lymphogranulomatosis, and in the fourth case a keloid-like tumour was found in the wound after removal of a basal-cell carcinoma Here, as in the preceding group, the interval from the previous operations was usually short, varying from 3 weeks to 6 months The following example will illustrate a typical history

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tural that the fine-grained lubricant material easily spreads in the organism, as is discernible in the rapid intra-abdominal spreading in post laparatomy peritonitis Thus it is not quite unexpected that this material enters the abdominal cavity through its normal openings, or leaves this cavity through them Both of such instances have been demonstrated in our cases In case No 14 (Table 1), a typical starch peritonitis was noted in a young woman not previously subjected to operation Starch-granules of identical chemical and physical properties could be demonstrated in the emulsion of the condomes used by her husband over a period of two years (*Saxen et al* 1963) Similar cases have been reported by *Paine & Smith* (1957), and *Wiggers* (1962), the authors being of the opinion that the starch-like material had originated from previous vaginal explorations The short interval between the exploration and the intra-abdominal reaction, as can be seen from the pictures published, could also be taken as indicative of contamination rather than a true ascending spreading of the material Typical contamination of granulation tissue overlying the ovaries is represented in the case illustrated in Fig 14

As regards the transmission of the starch powder from the abdominal cavity to its extensions, the following example (Case 13) is presented

Case 13 A 4½-year-old boy had fallen from the first floor on to frozen ground, and was admitted to the hospital following severe abdominal pains A laparatomy was performed immediately, and the surgeon suspected an intra abdominal rupture The whole bowel was thoroughly examined at operation, but no ruptures were observed The liver and the spleen were of normal appearance Enlarged lymph nodes constituted the sole positive finding The appendix was removed, and the patient left hospital one week later in good condition and free from symptoms

Two months later, the patient returned to the hospital, his right testis had been swollen for some time after the operation Examination revealed a tense scrotum with exudate, and a tumour-like nodule on the right testis At operation, this tumour was found to be a rather hard olive-sized nodule Microscopical examination disclosed fresh granulation tissue on the tunica vaginalis, together with an abundance of typical starch-granules in the tissue

---

Figs 3 4

Examples of a relatively fresh starch granuloma (Fig 3) and of an old lesion caused by the same material The granulation tissue in the fresh granuloma is highly vascularized and cellular with foreign body giant cells and granulocytes The old lesion consists of dense connective tissue with only a few giant cells and inflammatory cells Haematoxylin van Gieson Magnification × 150

HISTOLOGY

In most cases, the histological changes are typical of foreign body reaction, the picture varying in accordance with the age of the lesion, the nature of the basic disease, the site of the granuloma, and so on (Figs 1 and 2, Plate 1a, b)

Connective tissue proliferation is observable as either fresh, richly vascularized granulation tissue, or as dense, fibrous scar or keloid-like tissue (Figs 3 and 4) Reticulin fibres are abundant, and often arranged in a circle around the starch granules and giant cells In some cases, fibrinoid necrosis has been seen associated with histiocyte reaction; the picture then resembles changes in rheumatoid arthritis

Giant cells are frequently noted, although not as a constant finding, in older lesions they are often lacking These are of the common foreign-body type, with granules discernible in their plasma and in their neighbourhood (Fig 2), Plate 1d)

Inflammatory cells are mainly lymphocytes and eosinophilic granulocytes The abundant eosinophilic infiltrations around the granules and giant cells seem especially typical of granulomatous lesions caused by starch powder (Fig 5)

Starch granules are so far the only diagnostic feature The granules are 10 to 20 micron in diameter, and barely visible on slides stained by haemalum-eosin or other routine stains when examined under normal light (Fig 6, Plate 1c) Occasionally, a dark spot is discernible in the centre of a homogeneous particle They stain intensively with Lugol's iodine solution (Figs 7 and 8), and by the periodic acid Schiff-reaction (Figs 9 and 10, Plate 1e) In strong polarized light, they appear as spherulite crystals (Maltese cross) (Fig 8 and 10) The refractive index has been measured in some slides, and also from the BIO-SORB preparation, and found to be 1 538

In addition to these typical spherulite particles, smaller grains which exhibit great variations in size and shape are frequently seen in the granulomas They can often be identified as partially disintegrated starch particles (Fig 8), but the possibility of other foreign particles being present cannot always be excluded

---

Figs 5–6

Fig 5 Photomicrograph of a starch granuloma showing the abundance of eosinophilic granulocytes around the granules (not visible) and the giant cell Haematoxyl[illegible]

F[illegible]

5

6

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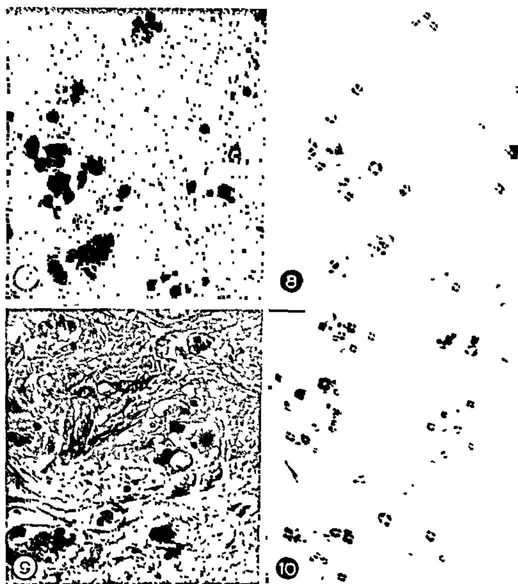
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Figs 5–6

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Fig [illegible] × 350 [illegible]



Figs 7–10

Sections from a starch granuloma stained with iodine solution (Fig 7) and with periodic acid Schiff reaction (Fig 9), and the corresponding sites photographed in polarized light The granules stain intensely blue with iodine whereas the P A S-reaction seems to stain only some of the starch granules in the section Magnification × 300

DIFFERENTIAL DIAGNOSIS

Abundant eosinophiles associated with a proliferation of atypical reticulum cells might lead to a suspicion of malignant lymphogranulomatosis; fibrosis, fibrinoid necrosis and a histiocytic reaction might result in thoughts of rheumatoid arthritis (Fig 4) Giant cells, epithelioid-like histiocytes and small granulomas can be indicative of tuberculosis, and some lesions cannot be distinguished from "sarcoidosis",

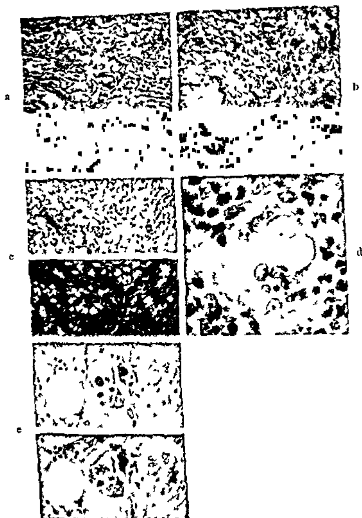


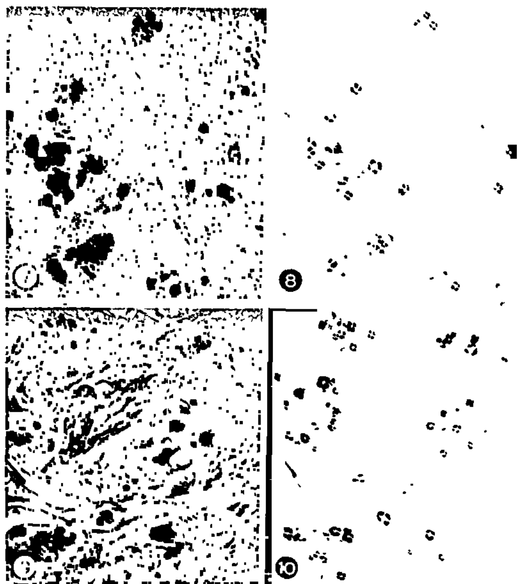
PLATE 1

Figs a ...

Fig ...

Fig d Two starch granules in the cytoplasm of a typical ...

Fig e ...



Figs 7 10

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Figs 12 13

Two examples of the presence of starch granules in metastatic lesions In Fig 12 the few atypical cells (upper center) are easily overlooked in the foreign body reaction caused by the starch powder In Fig 13 the main lesion seems to be the metastatic tumour and the granules have not caused any definite reaction Haematoxylin eosin Magnification × 250

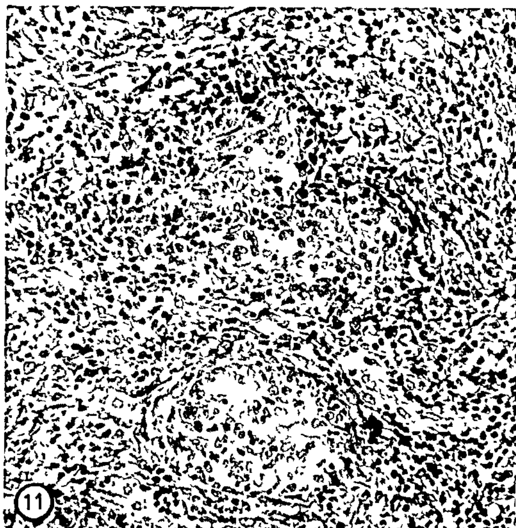


Fig 14

Fresh granulation tissue lining the ovaries of a woman not previously operated on shows two typical starch granules one of which seems to be buried deep in the tissue The total lack of any tissue response in the vicinity of granules indicates that this is contamination rather than a lesion caused primarily by the starch powder Haematoxylin eosin Magnification × 250

nation of the whole sample has led to a detection of the granules, which are furthermore frequently seen in partially disintegrated, hardly recognizable form

The *most important* practical point is that of malignancy in these



Figs 11
Starch-powder lesion with numerous giant cells and granulomas resembling the tissue reaction often referred to as sarcoidosis Haematoxylin eosin Magnification × 400

whatever this title may mean (Fig 11) The correct diagnosis can be arrived at by the detection of the typical granules in polarized light, although this too can be complicated by a number of factors

The commonest cause of misinterpretation is that the pathologist never gives a thought to foreign-body irritation, thus he does not use polarized light, but interprets the lesion as an unspecific granulation Even examination in polarized light does not always reveal the presence of the typical granules This can obviously be ascribed to the inadequate amount of light employed in the examination—the intensity which is comfortable for routine microscopy is often too low when a changeover is made to polarized light The other reason for failure to make the correct diagnosis is naturally a true lack of the granules, a result of their complete absorption In some older lesion, only a thorough exami-

the only indication in this direction being the local and sometimes generalized eosinophilia

Other factors involved in the manifestation of the lesions are also unknown It is possible as *A Saxen & Tuovinen* pointed out in 1947 that in some circumstances and at some sites the absorption of starch particles is delayed resulting in a prolonged irritation and the development of lesions Moreover other components added to the starch powders have to be taken into consideration as the possible causative agent in this case the visible starch granules would act as no more than some kind of marker The BIO SORB powder mostly used in Finland includes 2 per cent of magnesium oxide which according to *Seeling* (1948) was found to cause granulom and plaque formation and should therefore be omitted Against this *Lee et al* (1952) compared the irritative effect of starch powder containing either 2 per cent magnesium oxide or 3 per cent magnesium carbonate and could show no difference

As was pointed out above the contamination of tissue by starch powders seems to be rather common In addition to poorly washed and split gloves contaminated instruments and bandages as well as air borne infection must be borne in mind as possible sources From the pathologist s viewpoint it is important to remember this group of lesions Peritonitis following a laparatomy peritoneal carcinosis intra abdominal lesions resembling tuberculosis recurrences and persistent local inflammations constitute a group in which this lesion is to be found in daily routine An examination under strong polar...

... on and malignancy (metastasis) must be given some thought however and the detection of starch granules does not rule out such a possibility

SUMMARY

In the light of 14 cases of the authors and previous literature the problem of starch granulomas is viewed from the pathologist s point of view The typical histological features of this lesion are briefly described and the clinical data are discussed Three cases which represent different clinical courses are described in more detail a typical postlaparatomy starch peritonitis a case of recurrence and a case in which the substance spread from the abdominal cavity to the scrotum

Differential diagnosis and the misinterpretation of these lesions is discussed in greater detail The detection of typical spherulite bodies under polarized light seems to be the only diagnostic feature

lesions As was mentioned above, the gross appearance of the lesions is often indicative of either a peritoneal carcinosis or a local, recurring tumour Of course, the detection of starch granules in a lesion cannot exclude the possibility of a malignant lesion, two of the starch granuloma cases of the present series showed tumour tissue as well (Figs 12 and 13) Nevertheless, atypical reticulum cells often noted in starch granulomas, in conjunction with the mental influence exerted by the surgeons authoritative opinion, may lead to an erroneous diagnosis of metastasis Particularly in cases where the granules are not easily detectable, where the giant cells are absent, and the picture is characterized by proliferating connective tissue, this misinterpretation is near to hand

Erroneous positive diagnosis may result from two conditions Contamination of the sample during the biopsy is not too infrequent In case No 3 above, the specimen taken at the primary laparatomy was later re-examined, and found to contain typical contaminant granules (Fig 14)—subsequently, this contamination led to a typical starch granuloma Differential diagnosis between contamination and true starch reaction might be extremely difficult to achieve in samples of loose richly vascularized and proliferating granulation tissue Only by observation of the tissue structure around the granules is a correct judgement made Another condition which could lead to a misinterpretation is the presence of certain lipids in the tissue sample According to *Tison* (1953), some cholesterol compounds display the typical Maltese cross in polarized light, and only histochemical stainings make a differential diagnosis possible

DISCUSSION

The true frequency of tissue lesions attributable to starch powders is difficult to estimate Minor lesions, and even definite adhesions, do not always lead to a biopsy even if they do, the true causative agent is frequently overlooked by the pathologist, as put forward above Obviously a major proportion of these cases recover with no permanent adhesions, furthermore, old lesions which lack granules can no longer be diagnosed The patients of the present series have been followed for 5 to 24 months, and no further complications due to the glove-powder irritation have been observed In view of the wide use of these powders, and the fact that gloves are frequently split during operations (some statistics give the figure as in 75 per cent of them), it seems likely that only *some patients respond* to the starch powder (or some other components of the powder) An additional individual factor a susceptibility or allergy, is clearly required for the manifest lesion In one of our cases, the history indicated earlier allergic reactions towards other allergenes, but a skin test with BIO SORB was negative To data, no clear evidence is available to bear out the idea of an allergic reaction,

the only indication in this direction being the local and sometimes generalized eosinophilia

Other factors involved in the manifestation of the lesions are also unknown It is possible, as *A Saxén & Tuovinen* pointed out in 1947, that in some circumstances and at some sites the absorption of starch particles is delayed, resulting in a prolonged irritation and the development of lesions Moreover, other components added to the starch powders have to be taken into consideration as the possible causative agent in this case, the visible starch granules would act as no more than some kind of marker The BIO SORB powder mostly used in Finland includes 2 per cent of magnesium oxide, which according to *Seeling* (1948) "was found to cause granulom and plaque formation" and should therefore be omitted Against this, *Lee et al* (1952) compared the irritative effect of starch powder containing either 2 per cent magnesium oxide or 3 per cent magnesium carbonate, and could show no difference

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Erroneous positive diagnosis may result from two conditions. Contamination of the sample during the biopsy is not too infrequent. In case No. 3 above, the specimen taken at the primary laparatomy was later re-examined, and found to contain typical contaminant granules (*Fig.* 14)—subsequently, this contamination led to a typical starch granuloma. Differential diagnosis between *contamination* and true starch reaction might be extremely difficult to achieve in samples of loose, richly vascularized and proliferating granulation tissue. Only by observation of the tissue structure around the granules is a correct judgement made. Another condition which could lead to a misinterpretation is the presence of certain lipids in the tissue sample. According to *Lison* (1953), some cholesterol compounds display the typical Maltese cross in polarized light, and only histochemical stainings make a differential diagnosis possible.

DISCUSSION

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The Institute for Experimental Medicine (Head: Jens Bing, M.D.) and the Rheumatic Research Laboratory, Institute of Pathological Anatomy (Head: Gunnar Teilum, M.D.), University of Copenhagen, Denmark

SUBSTRUCTURES IN BASEMENT MEMBRANES OF THE RENAL MEDULLA STUDIED IN FREEZE-DRIED TISSUE

By

POUL FAARUP and HANS E. CHRISTENSEN

Received 5.xi.64

Electron microscopical investigations of the capillary basement membranes of the kidney have only given little information about substructures, when ordinary fixation of the tissue and conventional afterstaining methods have been used.

In the glomerulus a fine fibrillar structure has been demonstrated in the dense layer between the endothelial and epithelial cells (*Yamada* 1955, *Rhodin* 1955, *Bergstrand* 1957, *Kurtz & McManus* 1960) and, on the endothelial side of the dense layer, reticular fibrils were found in the glomerular membrane by *Farquhar, Wissig & Palade* (1961).

In recent years the use of various forms of cryotechnique has revealed new biological structures in different tissues and specialized organs (*Sjöstrand & Baker* 1958, *Fernandez-Moran* 1959, *Baker* 1962, *Hanssen & Herman* 1962, *Sjöstrand* 1963). It has been the aim of this work to make use of the technical advantages of this type of preparation to investigate the basement membranes in freeze-dried tissue from the renal medulla.

MATERIAL AND METHODS

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MATERIAL AND METHODS

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The findings were ...

Conference on
a grant from
Danish Associa

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The tissue and the crystals were then transferred to a chamber, the atmosphere of which contained some water vapour, which increased with time The temperature was later on changed several times between 4° C and 30° C After 1-2 weeks the tissue was placed in Palade's fixative (*Palade* 1952) in which the osmic acid concentration was changed to 4 per cent, the final fixation being performed in 6 hours The tissue blocks were imbedded in Vestopal W as described by *Zelickson & Hartmann* (1960), but the imbedding procedure was carried out in the course of several days since the infiltration of this kind of freeze dried tissue with the plastic seemed to proceed somewhat more slowly than in specimens fixed by the usual methods

When 1 2 microns thick sections from the tissue blocks in the light microscope showed areas of medullary capillaries from the *rete mirabile* and closely connected loops of Henle, sections of 300–600 Å were made on an LKB Ultratome The examination of the sections was performed in a RCA I MU-3B electron microscope or in a Siemens Elmiscop I Some of the sections were counterstained with a solution of 1 per cent uranyl acetate (*Strugger* 1956)

RESULTS

1 *The Capillaries of the rete mirabile*

In some of the medullary capillaries from 4 of the kidneys investigated, a well structured basement membrane was observed beneath very damaged endothelial cells Because of this cellular destruction, the identification of the capillaries was based on the finding of red blood cells in the lumen of the vessels Generally, serial sections were made of the structures investigated, so as to permit a more positive identification In the best preserved capillaries the subendothelial

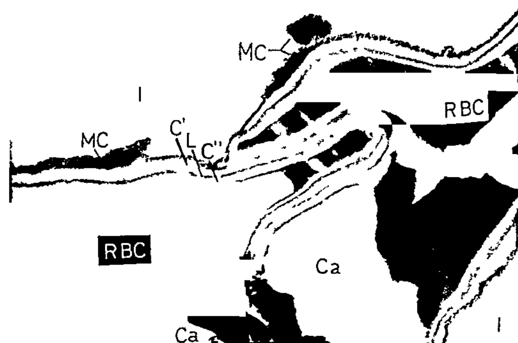


Fig 1

Low power view of two adjacent capillaries in the renal medulla (× 9 500)

Abbreviations used in the figures

Ca capillary RBC red blood cell TC tubular cell I interstitium MC mesenchymal cell C' light external layer L dense layer C'' light internal layer

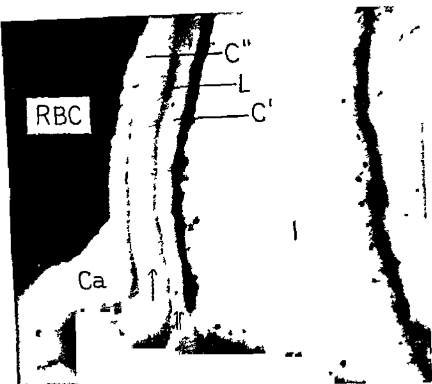


Fig 2

Cap llary basement membrane containing a dupl cation of the dense layer In the internal layer small granules (single arr v) and in the external layer larger granules (double arrow) are found Uranyl acetate (× 25 000)

basement membrane was seen to contain a well defined three layered structure (Fig 1) having a light internal layer a highly osmiophilic dense layer and a light external layer The total thickness of the base ment membrane was in most cases between ½ and 1 micron

The light internal layer was often found to have a homogenous appearance and a thickness of 0 3 0 4 micron Occasionally granules of a diameter of 200 300 A (*i e* corresponding to the size of large plasma protein molecules) were seen within it (Fig 2) In some of the vessels transversally oriented lines were found extending from the endo thelium and in the direction of the dense layer (Fig 3) Fibrils *e g* reticular fibers were never observed in this area in the freeze dried tissue

Lamina densa In the dense layer located near the middle of the base ment membrane two different substructures were observed In the great majority of the sections a transverse fibrillar structure was de monstrable (Figs 3 and 4) In the osmiophilic substance light pores or "channels were found having a diameter of 100 300 A usually of 200 A or less giving the area a sieve like appearance The channels



3

I

MC

C'

L

C''

Ca

4

may be seen sectioned obliquely in Fig 5 In one capillary there appeared to be a duplication of the dense layer (Fig 2) Here the external part of the dense layer was thinner and less osmiophilic than in the more centrally placed part The cross striation was observed in both of them

In a few cases the cross striation of the dense layer was replaced by four strongly osmiophilic layers (Fig 6) each having a thickness of about 70 A the space between them being about 50 A In these instances the demarcation of the dense layer from the internal and external layers was more distinct than when the dense layer had a sieve like appearance A mixture of the two types of substructures in the dense layer could also be found (Fig 7)

The light external layer showed the same scanty osmiophilia as the internal layer and sometimes contained granules which were found to be more heavily stained and usually of a somewhat bigger size than in the internal layer *i e* about 500 A (Figs 2 and 8) The thickness of the external layer was generally the same as that of the internal layer

The demarcation of the basement membrane from the interstitial space was made up of a strongly osmiophilic layer in which small projections were always found on the outher side

Mesenchymal cells in which the substructures of the darkstained cytoplasm were destroyed by the technique used were frequently found *attached* to the capillary basement membrane but apart from the points at which the mesenchymal cells were in contact with the capillary basement membrane no basement membrane like material surrounded the cells (Fig 1)

Sometimes the basement membrane was observed to be common to two adjacent capillaries (*Fig 1*) The external layer from the two vessels were thus fused together separating the dense layers of the two capillaries and giving rise to a more marked osmiophilia In these cases the external layers did not contain any granules but the sieve like structure of the dense layers was still visible (Figs 9 and 10)

2 *Basement Membranes of Tubular Structures*

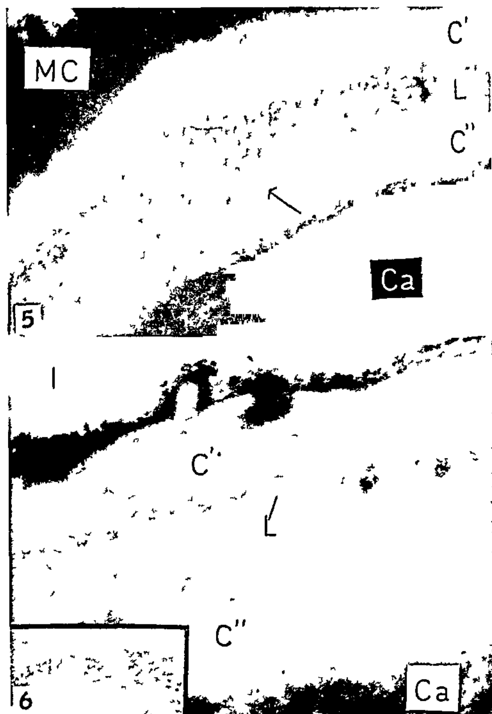
Basement membranes in which the substructures did not correspond to the foregoing description were also encountered In the lumen of these structures red blood cells were never found when using serial sec tions As in the capillaries the cells were found to be very much damaged

---

Figs 3–4

Fig 3 Capillary basem n m — ro w osm ophil c lines (ar to the dense layer in 000)

Fig 4 C st u u as a regular filter like (× 100 000)



Figs 5–6

Fig 5 Capillary basement membrane The filter like structure of the dense layer obliquely sectioned (× 75 000)

Fig 6 The laminated substructures in the dense layer of the capillary basement membrane consisting of four osmiophilic layers Uranyl acetate (× 85 000) Inset The four osmiophalic layers at a higher magnification (× 130 000)

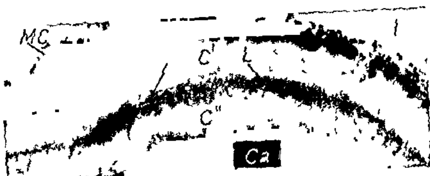
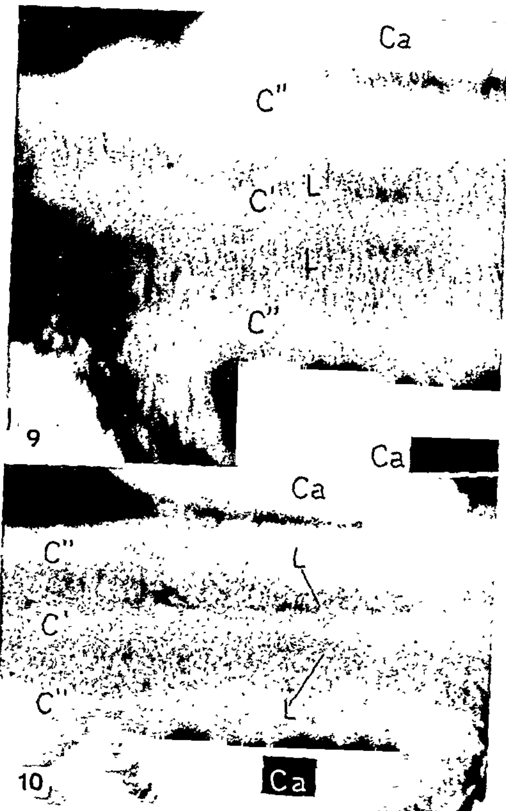


Fig 7
Capillary basement membrane In the dense layer a laminated structure (arrow) as well as a filter like structure are seen (× 40 000)

Fig 8
In the external layers of the capillary basement membrane (single arrow) and of the tubular basement membrane (double arrow) granules of the same size are f und (× 21 500)

and heavily stained and they contained no identifiable cell organelles (Fig 11) Because of the lack of erythrocytes it was concluded that these structures represented tubular portions of nephrons, *e g loops of Henle* As in the capillary basement membrane three layers could usually be identified in these structures (Figs 11 and 12) The internal layer was always seen to be homogenous in structure, but in the ex



Figs 9–10

Fig 9. Fused part of two capillary basement membranes *In both the dense layers* a filter-like structure is seen (× 100,000).

Fig. 10. Fused part of two capillary basement membranes, *in* which the pores in the dense layers are cut somewhat obliquely (× 71,000).

Fig 11
In the fused parts of the two tubular basement membranes the dense layers are absent (× 15 000)

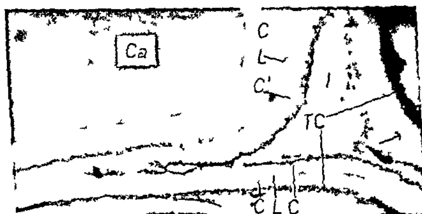


Fig 12
In the external layer of the capillary basement membrane and of the two tubular basement membranes granules of the same size are seen In one of the tubular basement membranes the dense layer is absent (arrow) (× 13 000)

ternal layer granules of the same size as those present in the corresponding part of the capillary basement membrane might be found (Figs 8 and 12) The total thickness of this basement membrane was about the same as that of the vessels

Normally the internal and external layers of the basement membrane were separated by a thin, osmiophilic line, which did not contain substructures corresponding to those in the dense layer of the capillary This line was absent in some of the basement membranes, especially when the external layers of two tubular structures fused together (Fig 11) In such cases the separation of the external and internal layer was marked by a slight difference in the osmiophilia of the two structures (*Figs 8, 11 and 12*) *Mesenchymal cells similar in appearance* to those observed in the case of the capillaries could also be found in close association with the tubular basement membrane

DISCUSSION

The above description of substructures of the basement membranes is quite different from observations made with the use of more conventional techniques The main problem in the evaluation of the results will be to determine to what extent artefacts are involved in both the filter-like structure and the laminated composition of the dense layer of the capillary basement membrane

It is well known that the application of freeze-drying to biological material may produce artefacts, *e g* cellular damage, as can be observed in the light microscope (*Meyersbach* 1956) as well as in the electron microscope This property is clearly reflected in the cellular destruction observed in our preparations However, the demonstration of the highly complex and specialized substructures of the basement membranes speaks strongly against the argument, that the varied morphology found is a result of the preparation itself, the difference between capillary and tubular basement membranes support the assumption of this being a biological phenomenon

In the freeze-dried tissue the total thickness of the capillary basement membrane was seen to be significantly greater than was the case in conventional preparations The observed filter-like structure of the dense layer found here seems to be compatible with observations concerning capillary permeability *Farquhar, Wissig & Palade* (1961), using ferritin as a tracer and conventional fixation technique, showed that in the glomerulus the dense layer of the capillary basement membrane acts as the main filter, although a filter like structure was not observed The ferritin particles *employed had an average diameter* of 100 A, which is somewhat less than the diameter of at least some of the channels in the dense layer observed in this study, this fits well with the observations of *Farquhar, Wissig & Palade*, according to which the filter was found to be imperfect The dimensions of the channels as

delineated by the osmiophilia need not however be identical with the physiologically active size of the pores

The occurrence of the two different types of basement membranes beneath the endothelial and tubular cells respectively as seen in Figs 8 and 12 supports the suggestion that the basement membranes are qualitatively different That the dense layer when present in basement membranes of tubular structures was seen as a thin line without the sieve like substructure found in the capillary basement membrane seems to be in accordance with the fact that the permeability of the two kinds of structures is of different nature The occurrence of morphological identical granules in the external layers of the two types of basement membranes could speak in favour of this layer at least being formed by the mesenchymal cells which are found in close apposition to the basement membranes (Figs 1 and 8)

SUMMARY

Tissue from the renal medulla of the mouse was freeze dried and investigated in the electron microscope The capillary basement membrane which was always seen as a well defined three layered structure consisted of an external and an internal light layer separated by the dense layer Two types of substructures were noticed in the dense layer a filter like structure and a laminated structure In the internal as well as the external layer of the basement membrane granular structures of different size could be found The basement membranes of tubular structures found in the same region of the kidney seemed to be qualitatively different from the capillary basement membranes the dense layer when present being without specialized substructures The data presented are in accordance with the assumption that the dense layer acts as the main filter of the capillary wall

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INFLUENCE OF THE THYMUS ON THYROXIN INDUCED LYMPHATIC HYPERPLASIA IN YOUNG GUINEA PIGS

1. *Treatment with Thyroxin one Month after Thymectomy*

By

ULF ERNSTRÖM

Received 10.xi.64

Thyroid hormones have a stimulating influence on the normal growth of the lymphatic tissue and its regeneration in guinea pigs (*Gyllensten* 1953, 1962, *Ernström & Gyllensten* 1959). Similar observations have been made in mice (*Marder* 1949, 1951), rats (*Cameron et al.* 1921, *Reinhardt et al.* 1942, *Shrewsbury et al.* 1955) and rabbits (*Marine et al.* 1924).

Studies by *Miller* (1961), *Jancovic et al.* (1962) and *Sherman et al.* (1963) in the mouse, rat and golden hamster have disclosed the importance of an intact thymus for the postnatal growth and immunological function of the lymphatic organs. It has also been suggested that in the adult mouse the thymus is concerned with immunity and lymphocytopoiesis, especially during regeneration after roentgen irradiation (*Miller et al.* 1963, *Lzar* 1963). In contrast to the findings in newborn mice and rats, thymectomy in newborn guinea pigs produces transient lymphatic hyperplasia during the first weeks after operation (*Gyllensten* 1953), but the consequences at later periods after thymectomy are not known.

An interrelation between the thyroid and the thymus is well documented. Thus, human hyperthyreosis is often combined with thymic hyperplasia (for ref. see *Matti* 1911, *Rathcke* 1937, *Adler* 1938, *Sarteschi* 1955). Experimental evidence of thymic hyperplasia in hyperthyreosis is most regularly found in young, well nourished animals treated with moderate doses of the thyroid hormone (*Gourrier* 1921, 1928, *Kluwanskaja-Kroll* 1929, *Carriere et al.* 1937, *Reinhardt et al.* 1942, *Feldman* 1951, *Aschkenasy* 1954). In the guinea pig, small doses of thyroxin are reported to stimulate growth of the foetal thymus (*Hoskins* 1910), as well as thymus regeneration after castration and thyroidectomy (*Comsa* 1951) and after irradiation-induced involution (*Gregoire* 1942). Treatment of the newborn guinea pig with the thyroid hormone results only in a slight and non-significant increase in the

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An interrelation between the thyroid and the thymus is well documented. Thus human hyperthyreosis is often combined with thymic hyperplasia (for ref. see *Matti* 1911, *Rathcke* 1937, *Adler* 1938, *Sarteschi* 1955). Experimental evidence of thymic hyperplasia in hyperthyreosis is most regularly found in young, well nourished animals treated with moderate doses of the thyroid hormone (*Courrier* 1921, 1928, *Khwanskaja Kroll* 1929, *Carriere et al.* 1937, *Reinhardt et al.* 1942, *Feldman* 1951, *Askenasy* 1954). In the guinea pig small doses of thyroxin are reported to stimulate growth of the foetal thymus (*Hoskins* 1910), as well as thymus regeneration after castration and thyroidectomy (*Comsa* 1951) and after irradiation induced involution (*Gregoire* 1942). Treatment of the newborn guinea pig with the thyroid hormone results only in a slight and non significant increase in the

thymic weight (*Gyllensten* 1953), which is also the case in the somewhat older guinea pig (*Ernstrom & Gyllensten* 1959). Massive doses of thyroxin produce atrophy of the thymus (*Andreasen* 1937, 1938) and accentuation of thymic atrophy in the castrated, thyroidectomized guinea pig (*Comsa* 1951). The influence of thyroxin on the cytology of the thymus indicates a stimulated cortical proliferation and probably also a stimulated migration of lymphocytes from the thymus (*Ernstrom* 1963).

This finding and the aforementioned recent thymus research raises the question whether thyrogenous lymphatic hyperplasia in the guinea pig is wholly or partly dependent on an intact thymus. In the present investigation, a comparison was made between the influence of thyroxin on the lymphatic tissue of thymectomized and sham operated guinea pigs.

MATERIAL AND METHODS

The 154 experimental animals were growing male guinea pigs weighing from 130–260 g at the start of the experiment. They were divided into four groups containing 41, 33, 39 and 41 guinea pigs respectively.

Group ST Sham operated. Thyroxin treated.
Group SC Sham operated. Control treated with saline.
Group TT Thymectomized. Thyroxin treated.
Group TC Thymectomized. Control treated with saline.

The guinea pig has a cervical thymus. Operation was performed under infiltration anaesthesia with 0.5 per cent Xylocaine® (Astra). The thymic lobes were mobilized at shamoperation and resected at thymectomy according to the technique used by *Gyllensten* (1953). Possible minor aberrant pieces of thymic tissue were not dissected and removed. However, no regeneration from such small remains was observed in any case. The cutaneous incision was closed with two silk sutures. Thymus resection was done as rapidly and as simply as the shamoperation; this is important in view of the fact that the lymphatic tissue is sensitive to stress.

Treatment with thyroxin or saline was started 31 days after operation. L-thyroxin sodium (Nyegaard & Co. A/S) in a dose of 50 μg/kg body weight was injected subcutaneously every 3rd day into the dorsum of the animals. The controls were given an equal volume of a 0.9 per cent solution of NaCl. The animals were killed 9 days after treatment was started (40 days after operation). The cervical, scapular, inguinal and mesenteric lymph nodes as well as the spleen and thymus were dissected quantitatively and weighed. The relative weight of the organs (mg/100 g body weight) was calculated. The results were analysed statistically by Student's *t* test.

RESULTS

Neither operation nor treatment influenced the general growth or gross appearance of the experimental animals. The weight of the relevant organs of the growing guinea pigs used in this experiment was linearly correlated to the body weight. All of the coefficients of correlation differed significantly from zero. The mean relative weights of the various organs are listed in Table 1.

Effect of thymectomy. The total lymph node mass was not significantly changed. The relative weight of the mesenteric nodes was decreased in the thymectomized guinea pigs as compared with the sham

TABLE 1

Relative Weight (mg/100 g Body Weight) of Thymus Lymph Nodes and Spleen of Guinea Pigs Treated with Thyroxin or Saline one Month after Thymectomy or Sham Operation Mean ± Standard Error

	No of animals	Thymus	Lymph nodes					Spleen
			Cervical	Scapular	Inguinal	Mesenteric	Total mass	
Sham operated thyroxin treated	41	104 81 ± 5 55	110 80 ± 4 81	24 85 ± 1 20	28 41 ± 1 17	140 36 ± 4 57	304 42 ± 7 35	140 20 ± 4 59
Sham operated control treated	33	105 50 ± 4 52	98 38 ± 3 69	23 53 ± 1 23	28 28 ± 1 47	131 57 ± 4 68	281 75 ± 7 48	125 99 ± 3 99
Thymectomized thyroxin treated	41		97 96 ± 3 57	21 93 ± 0 88	25 49 ± 1 28	124 38 ± 3 05	269 75 ± 5 40	134 93 ± 7 95
Thymectomized control treated	39		105 91 ± 4 53	24 77 ± 1 30	27 22 ± 1 56	119 00 ± 4 30	278 07 ± 7 87	134 94 ± 5 27

TABLE 2

Difference between Effect of Thyroxin on the Lympatic Tissue of Sham Operated and Thymectomized Guinea Pigse Relative Weight (mg/100 g Body Weight) of Lymph Nodes and Spleen Mean ± Standard Error

	Lymph nodes			Spleen
	Cervical and extremital	Mesenteric	Total mass	
Sham operated thyroxin treated / Sham operated control treated	13 89 ± 7 38	8 79 ± 6 54	22 67 ± 10 49	14 21 ± 6 99
Thymectomized thyroxin treated / Thymectomized control treated	13 72 ± 6 98	5 38 ± 5 36	—8 71 ± 9 57	—0 01 ± 9 54
Difference in effect of thyroxin	27 61 ± 10 16	3 41 ± 8 46	31 38 ± 14 20	14 22 ± 11 31
t	2 72	0 40	2 21	1 26
d f	150	150	150	150
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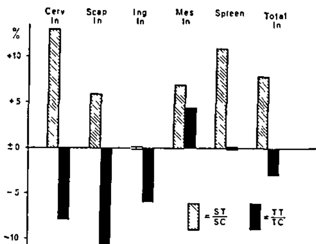


Fig 1

Comparison between effect of thyroxin on lymphatic tissue weight of sham operated and thymectomized guinea-pigs Hatched columns effect on sham operated animals black columns effect on thymectomized animals(effect in per cent)

operated controls The weight of the cervical nodes was, however, slightly increased The weight of the spleen was also increased None of the changes was significant

Effect of thyroxin on controls The total lymph-node mass was increased ($p < 0.05$) in the sham-operated guinea-pigs treated with thyroxin The increase in relative weight was most pronounced in the cervical nodes ($p < 0.05$), but was apparent in all the dissected groups of lymph nodes The weight of the spleen was likewise increased ($p < 0.05$)

Effect of thyroxin on thymectomized animals The total lymph-node mass was decreased but not significantly in the thymectomized animals treated with the thyroxin, as compared with thymectomized controls The relative weight of the cervical, scapular and inguinal lymph nodes was decreased ($p < 0.05$), whereas the mesenteric nodes showed a slight (non-significant) increase The relative weight of the spleen was unchanged

It is apparent that the stimulating influence of thyroxin on the lymphatic tissue of the sham-operated guinea-pigs was not reproduced in the thymectomized animals (Fig 1) On the contrary, thyroxin had a reducing effect on part of the lymphatic tissue of the thymectomized guinea-pigs in this experiment The stimulating influence of thyroxin on the total, dissected lymph node tissue was greater in the sham-operated than in the thymectomized animals ($p < 0.05$) As far as the cervical and extremital lymph-node mass is concerned, this difference in effect was still more pronounced ($p < 0.01$) As regards the mesenteric nodes, the difference in effect was not significant (Table 2)

Since thymectomy did not significantly change the total lymph-node mass (Table 3), it is also possible to compare directly the two groups

of thyroxin treated animals The total lymph-node mass of the thyroxin treated, sham operated animals was 13 per cent larger than that of the thyroxin-treated thymectomized guinea-pigs, this difference being highly significant ($p < 0.001$) · see Table 3

TABLE 3

Difference between Total Lymph Node Mass in Sham Operated and Thymectomized Guinea Pigs and in Thyroxin Treated Sham Operated and Thymectomized Guinea Pigs (mg/100 g Body Weight)

	Mean of difference	Standard error	*t* value	d f	Significance
Sham operated control treated / Thymectomized control treated	3.68	± 10.86	0.34	70	
Sham operated thyroxin treated / Thymectomized thyroxin treated	34.67	± 9.12	3.82	80	$p < 0.001$

DISCUSSION

The results demonstrate that the stimulating influence of the thyroid hormone on the lymph nodes is reduced one month after thymectomy Several alternatives to the rôle of the thymus in the genesis of thyroxin-induced lymphatic hyperplasia can be suggested

1 The increase in weight of the lymphatic organs after treatment with thyroxin is indirect, and mediated *via the thymus by a humoral* or cellular thymus factor

2 The stimulating effect of thyroxin on the growth of the lymphatic organs is direct, but wholly or partly dependent on the existence of a humoral thymus factor or a supply of thymogenic cells

3 The stimulating influence of thyroxin on the lymphatic tissue is direct and independant of thymus factors In the thymectomized animals *however*, an important source of lymphocytes is absent, and the remaining organs producing lymphocytes are forced to deliver more cells than in animals with an intact thymus This increased turnover of cells from the lymphatic organs of the thymectomized animals may conceal the stimulating influence of thyroxin.

In the present investigation, administration of thyroxin caused a decrease in weight of the different lymph nodes of the thy*mectomized* guinea pigs (except the mesenteric nodes) Thus, in addition to the known stimulating influence on growth, thyroxin seems to have a cell-depleting effect on the lymph nodes This mobilizing effect of thyroxin on the lymphocytes may be associated with its accelerating influence on catabolic processes *This is confirmed by* the established fact that the treatment with high doses of thyroxin results in lymphatic atrophy and general cachexia (*Ufferström* 1910, *Courrier 1921, 1928*, and personal observations) The observed decrease in weight of the lymph

nodes of the thymectomized guinea pigs treated with thyroxin may denote that the lymphocyte-depleting effect of thyroxin is much greater in the thymectomized animals than in the sham operated This may be due to the absence of both the normal and the thyroxin induced supply of cells from the thymus in the former animals All these lacking cells must be replaced by lymphocytes from the remaining lymphatic organs Simultaneously, the stimulating effect of thyroxin is absent, or is insufficient to result in a compensating proliferation and homeostasis in the lymph nodes and spleen of the thymectomized guinea-pigs

SUMMARY

Young male guinea-pigs were thymectomized or sham-operated, and treated with thyroxin (50 μg/kg body weight subcutaneously every 3rd day) or saline, from the 31st to 40th day after operation They were then killed, after which the cervical, scapular, inguinal and mesenteric lymph nodes, as well as the spleen and thymus, were dissected quantitatively and weighed The following observations were made

1 Thymectomy does not result in any significant change in the relative weight of the total lymph-node mass, nor of the individual groups of lymph nodes

2 Thyroxin treatment of sham operated animals produces an almost significant increase in the relative weight of the total lymph node mass The increase is most pronounced in the cervical lymph nodes The relative weight of the spleen is also increased almost significantly

3 Thyroxin treatment of thymectomized animals does not significantly increase the relative weight of the total lymph node mass The cervical and extremital lymph nodes are, on the contrary, decreased in relative weight

Statistically, the decreased stimulating effect of thyroxin on the lymphatic tissue after thymectomy is significant as regards the cervical and extremital lymph node mass, and evident but not significant as regards the mesenteric nodes and the spleen The total lymph node mass of the sham-operated guinea-pigs treated with thyroxin is 13 per cent greater than that of the thymectomized guinea pigs treated with thyroxin, this difference being highly significant

Thus, this investigation has shown that an intact thymus is important for the induction of lymphatic hyperplasia by means of thyroxin injections

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INFLUENCE OF THE THYMUS ON THYROXIN-INDUCED LYMPHATIC HYPERPLASIA IN YOUNG GUINEA-PIGS

2 *Treatment with Thyroxin Shortly After Thymectomy*

By

ULF ERNSTROM

Received 10 xi 64

It was found in a previous investigation that injection of thyroxin failed to induce lymphatic hyperplasia in guinea-pigs which had been thymectomized one month earlier (*Ernstrom* 1965) It was therefore concluded that an intact thymus is important for the induction of such hyperplasia It was suggested that the influence of thyroxin on the lymphatic tissue is either indirect and mediated by the thymus, or it is direct and only partly dependent on a humoral or cellular thymus factor If the effect of thyroxin on the lymphatic tissue is dependent on the thymus and completely mediated by it, the effect should differ in the thymectomized animals and in the controls immediately after thymectomy If the hyperplasia of the lymph nodes is partly dependent on thymus cells with a restricted lifespan after their delivery from the thymus, or by some *other thymus factor(s)* with similar *limitations*, there should be less difference in the effect of thyroxin treatment shortly after operation than one month after it

In the present investigation, thymectomized and sham operated guinea-pigs were injected with thyroxin shortly after operation A comparison was made between the effect of this treatment on the weight of the lymphatic organs in the thymectomized and the sham-operated animals The results were also compared with those obtained in similar experiments in which thyroxin treatment was given one month after thymectomy

MATERIALS AND METHODS

The experimental animal material consisted of 197 growing male guinea pigs weighing from 200–260 g at the start of the experiment They were divided into the following four groups

Group ST	Sham operated	Thyroxin treated
Group SC	Sham operated	Control treated with saline
Group TT	Thymectomized	Thyroxin treated
Group TC	Thymectomized	Control treated with saline

The experimental conditions were analogous to those in the previous investigation (*Ernström* 1965) except that treatment with thyroxin or saline was started *on the* 3rd day after operation and continued until the 12th day after this L-thyroxin sodium (Nygaard & Co A/S) in a dose of 50 µg/kg body weight was injected subcutaneously every 3rd day in the dorsum of the animals The controls were given an equal volume of a 0.9 per cent solution of NaCl The animals were killed on the 12th postoperative day The cervical scapular inguinal and mesenteric lymph nodes as well as the spleen and thymus were dissected quantitatively and weighed The relative weight of the organs (mg/100 g body weight) was calculated The *results were analysed statistically by Student's t test*

RESULTS

Thymectomy and thyroxin treatment did not influence the gain in body weight of the growing guinea-pigs used in this investigation The weight of the lymphatic tissue and the spleen was linearly correlated to the body weight The relative organ weights were compared (Table 1)

TABLE 1

Relative Weight (mg/100 g Body Weight) of Thymus Lymph Nodes and Spleen of Guinea Pigs Treated with Thyroxin or Saline Shortly after Thymectomy or Sham Operation Mean ± Standard Error

	No of animals	Thymus	Lymph nodes					Spleen
			Cervical	Scapular	Inguinal	Mesenteric	Total mass	
Sham operated thyroxin treated	50	149.23 +6.29	126.28 ±3.97	23.79 ±1.00	23.31 ±0.82	171.80 ±5.10	345.18 ±8.02	155.15 ±4.30
Sham operated control treated	48	144.32 ±5.48	121.16 ±4.01	22.20 ±0.67	24.05 ±0.91	164.53 ±3.88	331.96 ±6.50	153.46 ±6.39
Thymectomized thyroxin treated	44		118.92 ±5.11	22.11 ±1.07	23.39 ±1.25	163.11 ±5.28	327.53 ±9.57	148.63 ±4.75
Thymectomized control treated	55		121.46 ±4.01	23.37 ±0.97	22.80 ±0.73	150.10 +3.70	317.74 ±6.93	154.23 ±5.28

In the *thymectomized animals*, the total lymph node mass was decreased This change was due to a decrease in weight of the mesenteric nodes ($p < 0.01$)

Thyroxin treatment of the sham operated animals (group ST compared to group SC) increased the total lymph node mass The increase was most pronounced in the cervical nodes The weight of the spleen was almost unchanged

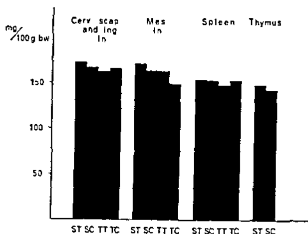


Fig 1

Relative weight of different parts of the lymphatic system in sham operated and thymectomized guinea pigs treated with thyroxin or saline shortly after operation
ST sham operated thyroxin treated SC sham operated control treated
TT thymectomized thyroxin treated, TC thymectomized control treated

Thyroxin-treatment of the thymectomized guinea-pigs (group TT compared to group TC) resulted in a small decrease in the weight of the cervical and scapular lymph nodes, but in an increase in the weight of the inguinal and mesenteric nodes The weight of the spleen was slightly decreased None of the changes was, however, significant

The effect of thyroxin on the sham-operated and the thymectomized animals was compared (comparison between the differences ST–SC and TT–TC, respectively) The increase in weight of the cervical and scapular lymph nodes in the sham-operated animals exceeded that of the corresponding nodes in the thymectomized animals (in fact, the latter did exhibit a decrease) This did not, however, apply in a com

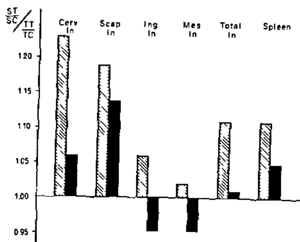


Fig 2

Comparison between the difference in effect of thyroxin on the lymphatic tissue of sham operated and thymectomized guinea pigs one month after thymectomy (hatched columns) and shortly after it (black columns)

parison between the effect on the inguinal and the mesenteric lymph nodes After thyroxin treatment weights of spleens were greater in the sham operated guinea pigs than in the thymectomized animals None of the differences in the effect was significant (Fig 1) The difference between the effect of thyroxin treatment on the sham operated and the thymectomized guinea-pigs in this investigation was compared with the corresponding difference in a previous experiment (*Ernstrom* 1965) It was then found that the difference between the reaction to thyroxin in the sham operated and thymectomized animals was much larger one month after thymectomy than shortly after this (Fig 2)

DISCUSSION

In the present investigation, treatment of the thymectomized animals with thyroxin shortly after thymectomy did not produce an increase in weight of the cervical and scapular lymph nodes and the spleen, as it did in the sham-operated animals The differences, however, are not significant Consequently, it has not been established that the effect of thyroxin on lymphatic tissue differs in sham operated and in thymectomized guinea pigs shortly after operation

If the present results are compared with those obtained when the hormonal treatment is started one month after thymectomy or sham operation (*Ernstrom* 1965), it is seen that the difference between the reaction to thyroxin of thymus resected animals and controls increased with the time interval between operation and institution of treatment (Fig 2) This finding argues in favour of the view that the effect of thyroxin on the growth of the lymphatic tissue and the spleen is not simply mediated by the thymus, but is only more or less supported by some humoral or cellular thymus factor Thus, the supply of thymogenic cells with limited life-span, or of lymphatic cells dependent on a thymus factor, may restrict the stimulating influence of thyroxin on the lymphatic tissue However, it is also possible that the effect of thyroxin is dependent on the thymus in a still more indirect way Thus thymectomy may result in a shortage of thymolymphatic cells, this shortage becoming more pronounced if a long time elapses after thymus resection The remaining lymphatic tissue is forced to deliver more cells, and the stimulating effect of thyroxin is abolished by the depleting effect

SUMMARY

Young male guinea pigs were thymectomized or sham-operated and treated with thyroxin (50 μg/kg of body weight subcutaneously every 3rd day) or saline, from the 3rd to 12th day after operation They were then killed after which the cervical, scapular, inguinal and mesenteric lymph nodes, as well as the spleen and thymus, were dissected quantitatively and weighed The following observations were made

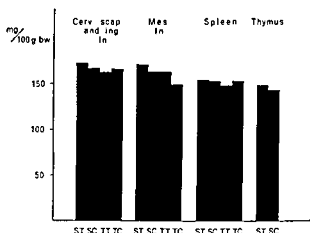


Fig 1

Relative weight of different parts of the lymphatic system in sham operated and thymectomized guinea pigs treated with thyroxin or saline shortly after operation
ST sham-operated thyroxin treated SC sham operated control treated,
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Thyroxin-treatment of the thymectomized guinea-pigs (group TT compared to group TC) resulted in a small decrease in the weight of the cervical and scapular lymph nodes, but in an increase in the weight of the inguinal and mesenteric nodes The weight of the spleen was slightly decreased None of the changes was, however, significant

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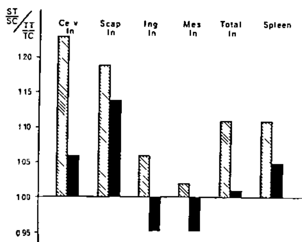


Fig 2

Comparison between the difference in effect of thyroxin on the lymphatic tissue of sham operated and thymectomized guinea pigs one month after thymectomy (hatched columns) and shortly after it (black columns)

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ANTIBODY RESPONSE AFTER "ATYPICALLY" PERFORMED SMALLPOX VACCINATION

By

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Received 26.x.64

As demonstrated in a previous study (5) the severity of local and general reactions after smallpox vaccination is quantitatively related to the vaccine potency. Accordingly, the use of a low potency vaccine was proposed for the vaccination of individuals in whom the risk of complication was supposed to be relatively high, e.g. in persons suffering from certain skin diseases (9).

The passive immunity provided by the injection of large doses of vaccinia immune gamma globulin (VIGG) has also been found to reduce the strength of reactions (5), and moderate doses of VIGG has been recommended for the prevention of complications (*Kempe* (8), *Nanning* (10)).

Finally, it has long been noted that newborn or very young infants exhibit mild responses to smallpox vaccination (*Wolff* (12), *Donally &*
...orschodt (4))
... nation reaction
... vaccination in
the presence of maternal immunity) represent a departure from the conventional technique of performing smallpox vaccination. Since they might be recommended for risk vaccinations, however, it would seem desirable to appreciate whether or not such procedures are equally effective as ordinary vaccinations. Before this question can be fully answered by investigating the *protective efficacy of the immunity* produced, some preliminary inference may be derived from serological studies.

The present report gives a brief account of the formation of neutralizing antibody in humans undergoing *modified* smallpox vaccination as mentioned above.

The authors are indebted to Mr. *Talmage Jacobs*, B.Sc., and Miss *Monica Handapik* for skilled technical assistance.

1 Thymectomy results in a decrease in relative weight of the mesenteric lymph nodes, whereas no pronounced change occurs in the rest of the lymphatic tissue

2 Thyroxin treatment of the sham-operated animals produces an increase in the total lymph-node mass, due essentially to an increase in weight of the cervical and mesenteric lymph nodes

3 Thyroxin treatment of the thymectomized animals results in a slight increase in the total lymph-node mass, due to an increase in weight of the mesenteric lymph nodes A slight decrease in weight of the cervical and scapular lymph nodes occurs

4 As far as the cervical and scapular lymph nodes and the spleen are concerned, the effect of thyroxin is greater in the sham operated guinea-pigs than in the thymectomized ones The difference, however is not significant

Thus, thymectomy does not significantly influence the induction of lymphatic hyperplasia by injection of thyroxin during the immediate postoperative period This is in contrast to the findings in a previous investigation, when thyroxin treatment was started one month after thymectomy

It is suggested that the thyroxin-induced lymphatic hyperplasia is not exclusively mediated by the thymus, but is potentiated by some cellular or humoral thymus factor Alternatively, the restricting influence of thymectomy may be due to a shortage of lymphatic cells

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experiments are presented to exemplify this (*Table 1*) *In the first, 500* tubes were inoculated with a small dose of vaccinia virus and then kept strictly stationary in the incubator Groups of 100 tubes were removed for reading of plaques after 2 3, 4, 5 and 6 days As seen in Table 1 the distributions of tubes according to numbers of plaques are very similar to the theoretical figures computed from the Poisson formula and therefore support the assumption that most plaques are primary lesions A similar test with half the number of tubes and a larger inoculum is given in the lower part of Table 1 In this test the tubes were moved once each day The figures indicate that, here again, most plaques were primary in origin during 3 days but later a secondary spread occurred (over representation of tubes with many plaques) On the basis of these results it was assumed that plaques read after 3 days were all primary if—as adapoted as a routine—tubes were kept strictly stationary

TABLE 1

Experiments to Show the Early Absence of Secondary Plaque Spread in Vaccinia Virus Infected Stationary (Standing) Tissue Culture Tubes The Table Gives Frequencies of Tubes with Specified Numbers of Plaques in Relation to Time after Inoculation of Small Amounts of Virus

	Plaques counted after	Proportions of positive tubes	Number of tubes with indicated number of plaques: Experimentally found distribution					Theoretically if no secondary spread is assumed (*Poisson distribution*)				
			0	1	2	3	>3	0	1	2	3	>3
Expt 1	2 days	13/100	87	12	1			87	12	1		
Inoculum about	3 days	30/100	70	25	5			70	25	4	1	
1 ID_{50} per tube	4 days	40/100	60	33	6	1		60	31	8	1	
Tubes not moved	5 days	56/100	44	36	18	1	1	44	36	15	4	1
before reading	6 days	57/100	43	43	11	1	2	43	37	15	4	1
Expt 2	2 days	23/49	26	16	6	1		26	17	5	1	
Inoculum about	3 days	38/50	12	16	12	6	4	12	17	12	6	3
2½ ID_{50} per tube	4 days	40/48	8	12	8	3	17	8	14	13	7	6
Tubes shaken	5 days	41/47	6	6	3	0	32	6	12	13	9	7
once every day	6 days	46/50	4	4	0	1	41	4	10	13	11	12

Empirical frequencies are compared to theoretical frequencies calculated (by the *Poisson formula*) from proportions of negative tubes assuming no secondary spread of plaques

The Neutralization Serum Dilution Curve

In the neutralization tests tenfold dilutions of serum were tested against a constant virus dose The fractions of surviving plaque units of virus (in per cent) were calculated and plotted against the log serum dilution on a probit form ...
give approximately st...
tion titres of the sera ... reproduci-
bility of this relationship in the present study is exemplified by data

MATERIAL AND METHODS

Vaccines One calf lymph vaccine, diluted about 10 times to a titre of $10^{6.5}$ $TCID_{50}$ per ml and in the sequel referred to as "weak vaccine", was used in group 1 below All other inoculations were done with 'ordinary vaccine', *i e* egg vaccine of a titre of about $10^{7.5}$ $TCID_{50}$ per ml

Vaccinia immune gamma globulin (VIGG) A preparation produced from pooled human postvaccination plasma by the National Bacteriological Laboratory and AB Kabi Stockholm was used throughout The vaccinia neutralizing titre was approximately the same as for some corresponding foreign preparations (Lister Institute Cutter)

Vaccinees vaccination and drawing of blood samples The study included the *following groups of individuals*

1) Adults suffering from different skin disorders or allergy, or presenting anamnestic data of such conditions They had not been previously vaccinated and were inoculated with the "weak vaccine" (titre $10^{6.5}$) Some of the subjects were given immune gamma globulin (VIGG) One venous blood sample for antibody titration was drawn from each individual at times varying from 1 to 20 months after vaccination mostly after 12 months

2) Adults and school children belonging to the same 'risk category' as group 1 They were all inoculated with ordinary vaccine (titre $10^{7.5}$) With respect to vaccination history and administration of immune gamma globulin (VIGG) the following subgroups were distinguished

a) Individuals undergoing primary vaccination

b) As group a) but receiving one (or occasionally two) injection of 5 ml VIGG at the time when 'take' had appeared

c) Persons undergoing revaccination giving a take

d) As group c) but treated with VIGG in the same manner as group b)

Sera were obtained 2½ 3 months after vaccination

3) Newborn infants vaccinated during the first week of life in a maternity ward. All mothers had been previously vaccinated as a rule more than 10 years ago From 16 infants giving positive reactions venous blood samples were drawn 4 6½ months after vaccination

4) A control group of healthy recruits and laboratory personnel reacting positively to revaccination with ordinary vaccine Blood samples were collected 1 2 months after vaccination

Neutralization tests Neutralization mixtures were prepared by mixing one volume of a clarified suspension of vaccinia virus (adjusted to give 10 50 plaques per 0 1 ml after 3 days incubation) with one volume of test serum appropriately diluted in Parker's medium 199 The mixture was incubated for 2 hours in a 37° C water bath and then inoculated in 0 2 ml amounts into emptied monkey kidney *culture* tubes grown out stationary (standing) After 2 hours of adsorption 1 0 ml of maintenance medium (Parker 199) with 5 per cent inactivated calf serum was added and the tubes were kept standing in a sturdy incubator at 36 centigrades for 3 days—Before reading one ml of a 1 per cent suspension of rooster erythrocytes was *added to each* tube At reading the number of haemadsorption foci were counted using a mounted lens with 8 × magnification—Sera were tested in 3 or 4 tenfold dilutions from undiluted or 1 10 Virus controls and a titration of a vaccinia negative serum as well as of a vaccinia positive standard serum were included in each test The per cent survival based on results from virus controls and plaque counts with negative serum were calculated and plotted against the log serum dilution in a probit scale The 50 per cent neutralizing titre was calculated through graphical interpolation between the survival percentages next below and next above the 50 per cent level

RESULTS

Estimation of the Degree of Secondary Spread of Viral Foci

Since the plaque neutralization tests were performed in culture tubes with a liquid medium, it was desirable to determine the period during which most viral lesions could be considered as primary plaques Two

Distribution of Neutralizing Antibody Titres in the Different Populations

The influence of vaccinia immune gamma globulin (VIGG) on the antibody formation was tested in groups of individuals after primary vaccination (group 2 a + b) and after revaccination (group 2 c + d) Unfortunately the indications for administration of VIGG had not been clearly defined in advance and hence the possible bias, depending on differences as to risk, age, vaccination history etc , cannot be estimated

The comparison between groups receiving vaccine alone and those given both vaccine and VIGG is shown in Table 2 The figures do not indicate any suppression of the antibody response by the injected VIGG

TABLE 2

Comparison of Neutralizing Antibody Titres Following Regular Vaccination and Vaccination Combined with Injection of Vaccinia Immune Gamma Globulin (VIGG)

		Number of individuals	Mean titer of neutralizing antibody (log units)	Standard error of mean titer	Difference
Group 2a	Primary vaccination	12	0 86	0 12	0 03*
Group 2b	Primary vaccination + VIGG	16	0 83	0 07	
Group 2c	Revaccination	16	1 60	0 14	−0 33†
Group 2d	Revaccination + VIGG	11	1 93	0 13	

* n.s.
† chance

The antibody response in a group of (group 1) inoculated with the weak of ordinary vaccine) is depicted in t average titre is at least as high as in the above mentioned group 2 a + b, plotted as a control group in the second row of Fig 2

The group of newborn infants (group 3) was assumed to exhibit a relatively high degree of maternal immunity since all the mothers presented evidence of successful vaccination in the past This was also reflected in the take frequency which was 67 per cent among the total number of 46 newborns in which vaccination was attempted (In a previous study (7) in 1 2 months old infants about 90 per cent takes were obtained with a vaccine of similar potency) The antibody titres $4\frac{1}{2}$ 6 months after vaccination in 16 of the positive newb are

The first three rows of Fig 2 represent individuals suffering from

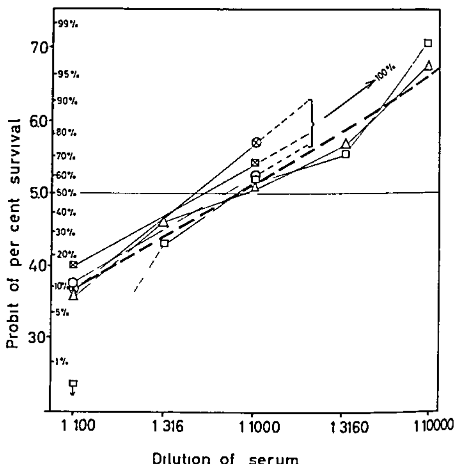


Fig. 1

Relationship between the dilution of a vaccinia positive standard serum and the degree of neutralization expressed as the fraction (per cent) of surviving vaccinia plaque units. Percentages are plotted on a probit transformed scale (ordinate). Data from five separate titrations are given. An approximate fit is indicated by the heavy, dashed line. Single serum titres were obtained by linear interpolation to 50 per cent survival.

from five repeat titrations of a positive standard serum presented in Fig. 1. In two of the titrations sera were tested at half-log dilutions and in three at one-log dilutions. The common fit of the points is approximately indicated by the heavy, dashed line. The slope of this line ($b = 1.5$) is of the same magnitude as found by *Cutchins et al.* With some few of the sera to be mentioned in the sequel only one dilution gave a per cent figure with a satisfactory weight (*e.g.* in the sequence <1 per cent, 37 per cent, 100 per cent). In such cases a line with the above slope was drawn through the reliable point and the titre read from the 50 per cent level.

As suggested in Fig. 1 there was some variation of the antibody titres obtained on different occasions. Accordingly an attempt was made in the several routine titrations to correct the titre values using as correction term the deviation of the titre of standard serum from the overall mean of that serum. However this correction resulted in an increased variability in all groups of vaccinees and was therefore considered unjustified. Consequently the following titres represent uncorrected values

Distribution of Neutralizing Antibody Titres in the Different Populations

The influence of vaccinia immune gamma globulin (VIGG) on the antibody formation was tested in groups of individuals after primary vaccination (group 2 a + b) and after revaccination (group 2 c + d). Unfortunately the indications for administration of VIGG had not been clearly defined in advance and hence the possible bias, depending on differences as to risk, age, vaccination history etc., cannot be estimated.

The comparison between groups receiving vaccine alone and those given both vaccine and VIGG is shown in Table 2. The figures do not indicate any suppression of the antibody response by the injected VIGG.

TABLE 2

Comparison of Neutralizing Antibody Titres Following Regular Vaccination and Vaccination Combined with Injection of Vaccinia Immune Gamma Globulin (VIGG)

		Number of individuals	Mean titer of neutralizing antibody (log units)	Standard error of mean titer	Difference
Group 2a	Primary vaccination	12	0.86	0.12	0.03*
Group 2b	Primary vaccination + VIGG	16	0.83	0.07	
Group 2c	Revaccination	16	1.60	0.14	−0.33†
Group 2d	Revaccination + VIGG	11	1.93	0.13	

* Difference not significant

† Probability ≈ 0.1 that difference is merely due to chance (Cf group 2 a d under Material and Methods)

The antibody response in a group of previously unvaccinated adults (group 1) inoculated with the weak vaccine (titre 10 × lower than that of ordinary vaccine) is depicted in the first row of Fig. 2. As seen, the average titre is at least as high as in the above mentioned group 2 a + b, plotted as a control group in the second row of Fig. 2.

The group of newborn infants (group 3) was assumed to exhibit a relatively high degree of maternal immunity since all the mothers presented evidence of successful vaccination in the past. This was also reflected in the take frequency which was 67 per cent among the total number of 46 newborns in which vaccination was attempted. (In a previous study (7) in 1–2 months old infants about 90 per cent takes were obtained with a vaccine of similar potency.) The antibody titres 4½–6 months after vaccination in 16 of the positive newborn infants are given in the fourth row of Fig. 2. On the average the titres are not significantly different from those found in the older groups of primary vaccinees (groups 1 and 2 a + b).

The first three rows of Fig. 2 represent individuals suffering from

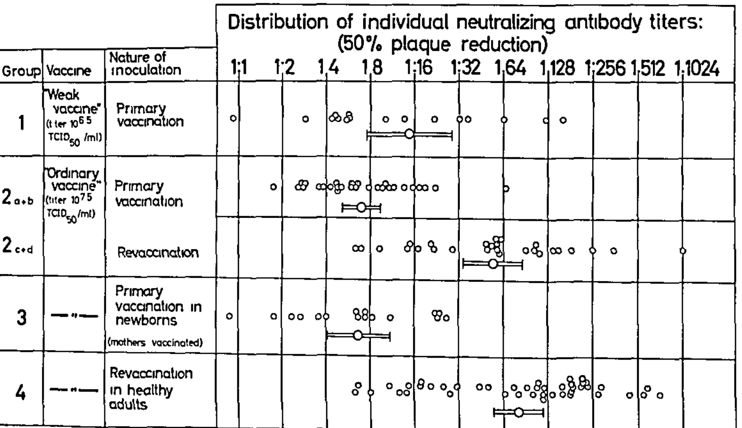


Fig 2

Distributions of individual titres of neutralizing antibody against vaccinia virus in "skin patients" (groups 1) subjected to primary vaccination with a weak vaccine (titre 10 × lower than that of ordinary vaccine) and in newborn infants (less than 1 week old) vaccinated with ordinary vaccine (group 3) Also titres of primary-vaccinated and revaccinated "skin patients' given vaccine of ordinary potency (group 2 c + d) and titres of a revaccinated control group of healthy individuals (group 4) are shown The mean and 95 per cent confidence interval of the mean of the titres is given for each group

various skin diseases, mostly eczema Earlier studies presented by Kempe (8) have suggested that such persons may exhibit a poor immune response to smallpox vaccination In the present study it was unfortunately not possible to obtain a sufficiently large material of healthy primary vaccinees, but only a representative group of revaccinated (last row of Fig 2) In comparison with this control group the revaccinated "skin patients" (group 2 c + d) displayed a normal pattern of antibody titres

DISCUSSION

The present study did not reveal any major reduction of the formation of neutralizing antibody after smallpox vaccinations in which certain precautions were taken to ensure attenuated reactions, i e by using a low titre vaccine, by injecting vaccinia immune gamma globulin (VIGG) after the take had appeared or by vaccinating in the presence of maternal immunity To be sure, the studied groups of vaccinees were small, with larger test group minor differences might have been demonstrated However, certain limits of the magnitude of possible true differences may be inferred from this study, and it may be stated that such differences are small, if at all present

In antigen dose-antibody response studies with other antigens (cf *Stevens* (11)) a linear relation has often been found between antibody titre attained and the square root of the antigen dose For smallpox vaccination however in which such a relationship has not been yet demonstrated, the immunizing antigen dose is the amount of viral substances produced in the pock, rather than the inoculated dose Though a certain correlation between immunizing dose and inoculated dose is probably present (5), the regression of the former dose upon the latter must be assumed to be relatively flat It might accordingly be speculated that for instance, a tenfold decrease of the inoculated dose would correspond to a smaller than $\sqrt{10}$ fold decrease of the antibody titre attained In agreement with this a relative independence of the antibody response of the vaccine potency was also demonstrated by Cross (1) in a small scale experiment

As to the effect of passive immunity it was shown in another study (6) that the presence of maternal antibody at a relatively high titre in one-month old infants did not exert any pronounced inhibition of the formation of neutralizing antibody The same was seen in the present study in newborn infants It is therefore very improbable that a 5 ml dose of immune gamma globulin, which in an adult human would be diluted more than 1000 fold and thus give a titre much lower than that found in the infants, would inhibit the antibody formation The conclusion to be drawn from this study is that there seems to be no disadvantage, as far as [illegible]
potency vaccine [illegible]
recommended [illegible] reduce the hazard of certain "risk

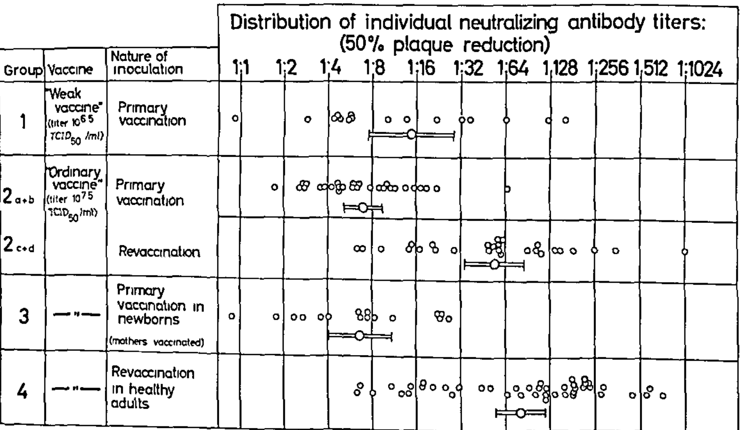


Fig 2.

Distributions of individual titres of neutralizing antibody against vaccinia virus in "skin patients" (groups 1) subjected *to primary vaccination* with a weak vaccine (*titre 10 × lower than that of ordinary vaccine) and in newborn infants* (less than 1 week old) vaccinated with ordinary vaccine (group 3) Also titres of primary vaccinated and revaccinated "skin patients" given vaccine of ordinary potency (group 2 c + d) and titres of a revaccinated control group of healthy individuals (group 4) are shown The mean and 95 per cent confidence interval of the mean of the titres is given for each group

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The Public Health Research Institute of The City of New York, Inc New York
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THE FORMATION AND PROPERTIES OF POLIOVIRUS NEUTRALIZING ANTIBODY[1]

5 *Changes in the Quality of 19S and 7S Rabbit Antibodies Following Immunization*

By

SVEN ERIC SVEHAG[*]

Received 2 xi 64

It was reported in a previous communication (1) in this series that early immune (days 1 and 2) and normal rabbit antibodies to polio virus (PV) were similar in their physicochemical properties as well as in their affinity for antigen Both these antibodies formed easily dissociable complexes with the viral antigen and both were macroglobulins which in zone electrophoresis migrated as nearly homogeneous components in the β region (1, 2) In contrast, the predominating 19S antibody from day 3 on was a γ_1 globulin which combined more firmly with the virus

The present report is an analysis of the affinity of 19S and 7S antibodies for antigen and the correlation between affinity and electrophoretic mobility In sera collected 2 3 weeks after immunization the 19S and 7S antibodies were of high and similar combining strength Late immune rabbit sera (IRS) contained predominantly 7S γ_2 globulin antibody, which formed very firm complexes with PV 7S antibody of low affinity was not found

MATERIALS AND METHODS

Virus Type 1 (Brunhilde) poliovirus was used Virus was cloned and grown on HeLa monolayers and virus stocks were partly purified and concentrated by differential *centrifugation prior to their storage* at —20° C The virus stock used in all dissociation experiments contained about 1 μg protein per $10^{9.4}$ plaque forming *units (PFU)*

Immunization procedure Healthy 3 ½ to 5 months old female randomly inbred Chinchilla Flemish rabbits weighing 2-½ to 3 ½ kg were injected with diluted

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[1] This investigation was supported in part by *Research Grant E 4360* from the *National Institute of Allergy and Infectious Diseases United States Public Health Service*

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vaccinations' There is also evidence that newborn infants vaccinated in an emergency situation, as in this study, exert a satisfactory antibody response At any rate they may be considered as having received basic immunization

SUMMARY

The formation of neutralizing antibody was investigated in a group of individuals after primary smallpox vaccination with a low potency vaccine, *in persons given vaccinia immune gamma globulin after vaccination*, and in newborn infants assumed to possess maternal immunity The results did not suggest that the antibody response in any of these groups was less efficient than in individuals vaccinated under normal conditions

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By

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stock virus (2 ml) into the marginal ear vein Three immunization schedules were used Group 1 (4 animals) received a single injection of 3×10^9 PFU, group 2 (4 animals) two injections of 7×10^8 PFU, spaced 40 days apart and group 3 (4 animals) four injections of 3×10^8 PFU with 2 weeks intervals The rabbits were bled by cardiac puncture the blood allowed to clot and the sera collected and stored at —70° or 3° C without preservatives

Assay of Virus Neutralizing Antibody, Zone Density Gradient Ultracentrifugation Zone electrophoresis and Protein Determination These methods were described in previous papers (2, 3) in this series

Dissociation of Virus Antibody complexes at acid pH Mixtures of virus (same stock in all experiments) and serum or virus and isolated 19S or 7S antibodies were incubated for 20 hours at 3° C and 3 hours at room temperature Aliquots of the mixtures were then diluted two fold in buffered salt solutions (BSS) of various pH The buffer solutions had been prepared so that the pH of the diluted reaction mixtures would range from about 2 5 to 6 5 The samples were left at room temperature for 40 minutes and the pH subsequently adjusted to neutrality by the addition of 14 or 19 volumes of ice cold neutral BSS When dissociation of virus antibody complexes was carried out at pH levels between 2 and 3 further adjustment with minute amounts of 0 1 M NaOH was required The virus serum mixtures were then immediately assayed on HeLa monolayer cultures (4 plate cultures/ sample)

RESULTS

Affinity[1] of Antibody Estimated by Dissociation[2] of Virus-Antibody Complexes at Acid pH

1 *Stability of 19S antibody at acid pH* The efficiency of acid dissociation in reactivating virus antibody complexes at different pH levels was taken as a measure of the affinity of the antibody in question To test the assumption that virus reactivation was due to the dissociation of virus-antibody complexes and not to antibody denaturation or denaturation and subsequent dissociation, the stability of rabbit antibodies at acid pH was studied

Fig 1 illustrates results obtained when normal sera and early IRS, in which only 19S antibody was detectable, were exposed to acid pH at room temperature for 1 hour Control serum samples were kept at the same temperature and neutral pH for an equal time Following incubation, the pH of all samples was readjusted to neutrality and the sera were simultaneously assayed for neutralizing activity to PV The results suggest that the normal 19S antibody was slightly more sensitive to high hydrogen ion concentrations than the early immune 19S antibody Incubation at the lowest pH levels employed in the majority of acid dissociation experiments (2 4–2 5) resulted in a loss of 20–40 per cent of the antibody activity At pH levels above 2 5 the reduction in antibody activity was barely measurable Since in all reactivation

1 *The term affinity as used here refers to the firmness of the virus antibody* ... with which infectious virus can be recovered
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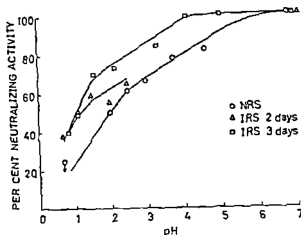


Fig 1

The stability at acid pH of normal and early immune poliovirus neutralizing anti bod es of rabbit or g n The sera were kept at the indicated pH levels and room temperature for 1 hour Only 19S antibodies were demonstrable in these sera by zonal dens ty grad ent centrifugation in sucrose The immune sera were collected after a single intravenous injection of 3×10^9 PFU of virus
○ normal serum △ 2-day immune serum □ 3 day immune serum

experiments most of the virus was recovered at pH levels above 2 5, denaturation of antibody did not contribute significantly to the reactiva tion of virus bound to antibody The stability at low pH of the 7S rabbit antibody in a 35 day serum was similar to or slightly higher than that of the 19S antibody It was subsequently found that antibody dissociated from virus also retained most of its original neutralizing activity Consequently the sensitivity to high hydrogen ion concentrations of antibody apart from or in combination with PV appeared to be the same

2 *Effect of antibody multiplicity on the efficiency of virus reactiva tion* A concentration of antibody which neutralized about 90 per cent of an estimated 5000 PFU of PV was employed as a standard dose in the acid reactivation experiments Because unexpected variations in

variations

results ob

19S antibody isolated by zonal density gradient centrifugation from this serum Only 19S antibody was de monstrable in this serum

The virus reactivation obtained at each pH increment is given in per cent of neutralized virus on the ordinate as noncumulative values The 1 20 dilutions of whole serum and the 1 2 dilution of isolated 19S antibody corresponded to approximately 2 5 antibody equivalents (estimated from virus survival levels reference 6) while the 1 5 dilu tion of isolated 19S antibody corresponded to about 1 equivalent The antibody multiplicity affected the reactivation curves the higher multi

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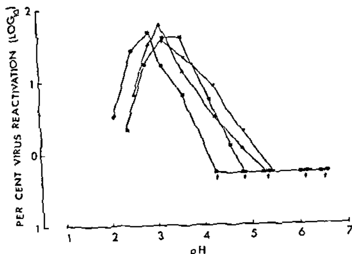


Fig 3

Effect of antibody multiplicity on the reactivation of poliovirus at acid pH Four different multiplicities of 19S antibodies isolated from a 7 day serum were used The rabbit had received a single intravenous injection of 3×10^9 PFU of virus ▼ 2 2 antibody equivalents ■ 4 1 antibody equivalents ▲ 4 5 antibody equivalents ● 6 2 antibody equivalents

between the location of the peaks of the distributions and the number of antibody equivalents employed

3 *Correlation between the affinity of an antibody and the time of its production* The sera of about 10 per cent of unimmunized rabbits were found to contain low levels of specific background neutralizing activity to PV The physicochemical properties of this ' normal antibody were similar or identical to those of the early immune macroglobulin antibody (1) Further the average free energy of binding was the same (~ -9 kcal mole) for the reaction between PV and normal or 2-days immune antibody It can be seen in Fig 4 that also the antigen binding capacities of these two antibodies were similar when measured by the pH method the 2 day serum containing some antibody of slightly higher combining strengt The χ^2 test revealed that the reactivation data obtained with the normal antibody (●) were normally distributed ($\chi^2_{(3)} = 4\ 7a$ P $\sim$ 0 2) A minor difference in antibody multiplicity caused no displacement of the reactivation curves The 2 day serum was obtained from a rabbit devoid of normal antibody as the presence of such antibody could obscure the results when studying the affinity of the early immune antibody

Fig 5 illustrates results obtained with sera collected from the same animal at 2 3 4 7 and 3a days following a single intravenous injection of 7×10^8 PFU of virus About 2 antibody equivalents were used For the 2-day IRS the percentage virus reactivation at different pH levels

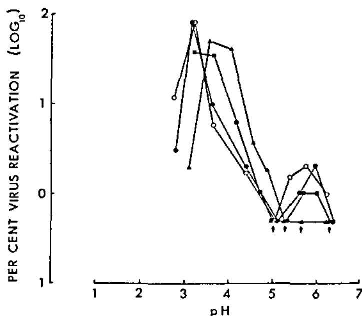


Fig 2

Per cent reactivation at acid pH of poliovirus neutralized by 3 day immune rabbit serum and 19S antibodies isolated from this serum ● serum diluted 1 20 in BSS, ○ serum diluted 1 20 in 30 per cent sucrose, ■ isolated 19S antibodies diluted 1 2 in 30 per cent sucrose, ▲ isolated 19S antibodies diluted 1 5 in 30 per cent sucrose The rabbit had received a single intravenous injection of 3 × 10^9 PFU of virus

plicity requiring a 2–3 fold higher hydrogen ion concentration in order to achieve the same efficiency of virus reactivation The slight discrepancy between the results obtained with whole serum and isolated 19S antibody (same antibody multiplicity) may be due to the participation of non-antibody serum components which are eliminated in the isolation of 19S antibody The type of diluent (30 per cent sucrose or BSS) used for the serum appeared to have no effect on the reactivation curves The 30 per cent sucrose solution was tested since the 19S antibody was recovered at about this sucrose concentration in the density gradient and in a few experiments was used undiluted The biphasic nature of the curves was of considerable interest since it indicated the presence of two 19S antibody populations of distinctly different average affinity

As only 1 to 2 per cent virus reactivation was observed at pH values between 5 2 and 6 2 the significance of this reactivation was tested in pooled t-tests In these tests P ranged between 0 30 and 0 05 However, when the data obtained at these pH values were paired the reactivation in this pH region was significant (P $\simeq$ 0 01)

Fig 3 illustrates a similar experiment in which four different multiplicities of 19S antibody, isolated from a 7-day IRS, were employed The use of increasing antibody concentrations caused a slight displacement of the reactivation curves toward more acid pH Note the relationship

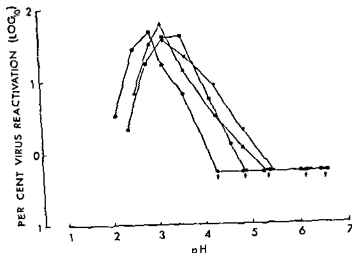


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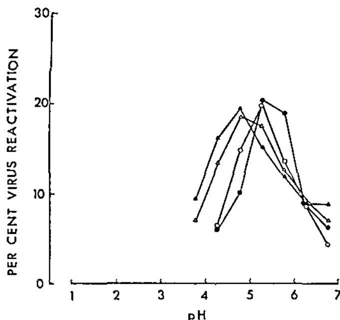


Fig 4

Per cent reactivation at acid pH of poliovirus neutralized by normal and early immune rabbit antibodies The normal and early immune antibodies were obtained from two different animals The immune serum was collected 2 days after a single stimulation with 7 × 10⁸ PFU of virus ○ normal serum 1 9 antibody equivalents ● normal serum 2 3 antibody equivalents, △ immune serum 2 0 antibody equivalents ▲ immune serum 2 5 antibody equivalents

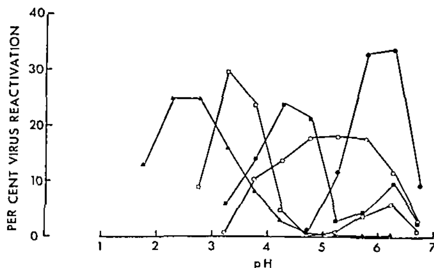


Fig 5

Per cent virus reactivation as a function of pH and the time of collecting the immune serum The sera were obtained from the same rabbit following a single intravenous injection of 7 × 10⁸ PFU virus ● 2 day serum ○ 3 day serum ■ 4 day serum □ 7 day serum ▲ 35 day serum

followed a nearly symmetric curve indicative of an approximately normal distribution of antibody of low affinity It may be seen that the average affinity of the 2 day IRS in Fig 4 was intermediate to that of the 2 and 4 day IRS depicted in Fig 5 The 2 day sera were obtained from two different rabbits which had both received 7×10^9 PFU of PV Thus the discrepancy may be due to a difference between individual rabbits or possibly to the fact that the serum in Fig 4 was collected slightly more than 2 days after immunization The skewed distribution curves obtained with the 2 day serum in Fig 4 suggest that synthesis of a more firmly combining 19S antibody had already begun The 3 day serum (Fig 5) gave a reactivation curve with a very broad base suggesting the rapid appearance of antibody of higher affinity The resolution was however insufficient to allow a separation of possibly existing antibody populations of different antigen binding capacity Two distinctly dissimilar populations were present in the 4 day serum Furthermore the predominating antibody in this serum was of considerably higher affinity than the 19S antibody synthesized 2 days earlier In the 7 day serum there remained only a trace of the weakly combining 19S antibody and it is likely that this trace amount consisted of antibody produced mainly during the first 2–3 days after immunization For the 35 day serum which contained predominantly 7S antibody the reactivation curve showed a slight positive skewness possibly indicative of a continued trend toward synthesis of even more firmly combining antibody The average affinity of the antibody in the 35 day serum corresponded to a hydrogen ion concentration as high as 10^{-5} M

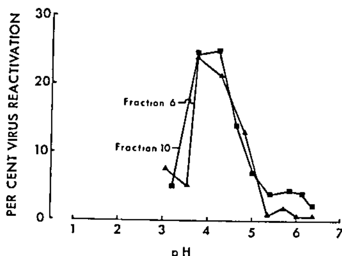
4 *Relationship between the net charge and affinity of antibodies* The increase in affinity of 19S antibody on day 3 coincided with an earlier described (2 7) shift in the electrophoretic mobility of 19S antibody ($\beta \rightarrow \gamma_1$) It was not known however whether th e changes

Thus it

different

ity from the same serum

A 4 day IRS was subjected to zonal density gradient centrifugation the 19S antibody recovered and its electrophoretic distribution determined by starch block zone electrophoresis (Fig 6 bottom part) The antibody regained from two regions (slow γ_1 and γ_1–β) of the block was concentrated by dialysis against Carbowax 20-M (Union Carbide Chemicals Company New York) dialysed against BSS and incubated with PV overnight at 3° plus 3 hours at room temperature After allowing virus and antibody to combine at room temperature the various virus antibody complexes containing 19S γ_1 and 19S γ_1–β globulins were subjected to acid dissociation The antibody multiplicities used were higher than in most other experiments for the 19S γ_1 globulin antibody about 6 and for the 19S antibody recovered in the γ_1–β region 4 0 For both 19S antibodies the reactivation curves were however, similar to the one obtained with the whole 4 day serum (Fig 5) The



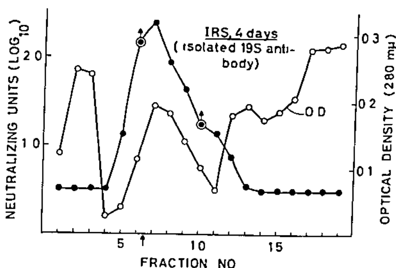


Fig 6

Per cent reactivation at acid pH of poliovirus neutralized by 19S antibodies of different average net charge (top figure) The serum was collected 4 days after a single intravenous injection of 7 × 10^8 PFU of virus and the 19S antibodies isolated by zonal density gradient centrifugation prior to determining their electrophoretic distribution by starch block zone electrophoresis (bottom figure) IRS, immune rabbit serum

γ_1-β-globulin fraction appeared to contain a trace of antibody of lower affinity reactivable with 10^{-5} –$10^{-6.0}$ M hydrogen ions This finding was of particular interest since it suggested that the weakly combining antibody observed in the whole 4-day serum (Fig 5) was associated with γ_1- and β-globulins This agrees with the observation that the 19S antibody of low affinity produced during the first 2–3 days after immunization was of high electrophoretic mobility (2)

5 *Affinity of isolated 19S antibodies* It can be seen in Fig 2 that 19S antibodies isolated from a 3-day serum gave a biphasic reactivation

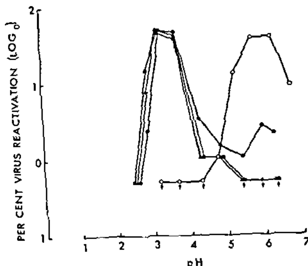


Fig 7

Per cent reactivation at acid pH of poliovirus neutralized by 19S antibodies produced at various times after immunization. The sera were obtained from the same rabbit following a single intravenous injection of 3×10^9 PFU of virus ○ 2 days whole serum ● 5 days isolated 19S antibody ■ 7 days isolated 19S antibody ▲ 14 days isolated 19S antibody

curve indicating the presence of two 19S populations of different affinity Similar biphasic curves were obtained with 4 day sera (Fig 5) The results with isolated 19S antibodies in Fig 7 indicate that the weakly combining antibody component in 3 to 5 day sera was of the same average affinity as the 19S antibody in the 2 day serum There fore the weakly combining 19S antibody recovered in the $\gamma_1 \beta$ region and produced mainly during the first 2–3 days after immunization appeared to be rapidly overshadowed on the 3rd and 4th day by a 19S antibody of distinctly higher affinity Between day 5 and 14 (Fig 7) this tightly binding 19S γ_1 globulin antibody showed no further increase in combining strength A comparison with Fig 8 reveals that the antigen binding capacity of the 19S antibody in 7 and 14 day sera was similar to that of the 7S antibody produced during the same period No 19S antibody of low affinity was demonstrable in the 8 and 14 day sera in this experiment

6 *Affinity of isolated 7S antibodies* 7S antibodies were isolated by zonal density gradient centrifugation from sera collected at various time intervals after a single intravenous injection of 3×10^9 PFU of virus Almost identical reactivation curves were obtained with 7S antibodies from 7 and 14 day sera (Fig 8) No measurable virus reactivation was observed between pH 5 and 6 with 7S antibodies isolated from these two sera If the earliest appearing 7S antibody on day 4 and 5 had been of low affinity some reactivation should have occurred in this pH region

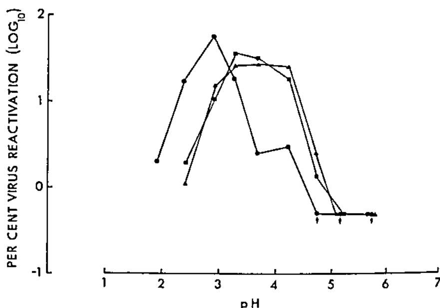


Fig 8

Per cent reactivation at acid pH of poliovirus neutralized by 7S antibodies produced at various times after immunization The sera were obtained from the same rabbit following a single intravenous injection of 3 × 10^9 PFU of virus and the 7S antibodies isolated by zonal density gradient centrifugation
▲ 7-day serum, ■ 14 day serum, ● 220 day serum

since the 7S antibody had a half-life of 7–9 days (3) Similarly, there was no virus reactivation at these pH values when 7S antibody produced later in the course of immunization was employed Thus, a 7S antibody of low affinity was not demonstrable

Many of the sera used in reactivation experiments were subjected to zone electrophoresis (2) In the 7-day serum (Fig 8) the 7S antibody was found to be associated with γ_1-globulins only, in the 14-day serum with predominantly γ_1- but also some γ_2-globulins The 7S antibody isolated from the 220-day serum (Fig 8) was a γ_2-globulin Antibodies of the 7S γ_2-globulin type were found to bind very firmly to the viral antigen but it was not investigated whether this was related to their lower net charge or to some other property acquired later in the course of immunization The inability to demonstrate weakly combining antibody (19S or 7S) in late sera was not due to too short a reactivation time (40 minutes) since 19S antibody of low affinity in early sera was dissociated in 15 minutes But one cannot exclude the possible existence in late sera of trace amounts of weakly combining antibody which may be difficult to detect in the neutralization test because of the competition for antigen from antibody of high affinity However since the neutralization test was conducted in antigen excess and the antigen-antibody reaction allowed to reach approximate equilibrium it appears unlikely that even minor amounts of slowly reacting antibody of low affinity escaped detection

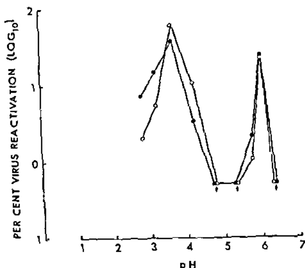


Fig 9

Per cent reactivation at acid pH of poliovirus neutralized by 19S antibodies The sera were collected from two different rabbits 2 days after a second intravenous inject on of 7 × 10⁹ PFU of virus. The time interval between the two injections was 40 days The 19S antibodies were isolated by zonal density gradient centrifugation

7 *The effect of antigenic restimulation on the affinity of 19S antibody* It was previously reported that subsequent to restimulation with PV antigen 19S antibody formation was promptly increased (8) The results in Fig 9 were obtained in an experiment with sera from rabbits that had been restimulated with PV (7×10^9 PFU) 40 days after the primary stimulus No loosely combining 19S antibody was demonstrable just prior to restimulation In contrast biphasic reactivation curves were obtained with sera collected 2 days after antigenic restimulation, indicative of a renewed formation of 19S antibody of low affinity

DISCUSSION

The present report has sought to (a) analyse the sequential changes in antibody affinity which occur with time after antigenic stimulation and (b) relate these changes to the production of different molecular types of antibodies Some of these findings have been described earlier in abbreviated form (1, 7)

The term "avidity was introduced by *Kraus* (9) to describe a qualitative property of diphtheria antitoxin The term has since been used with different connotations by different workers (9–16) The term "non avid sera is used for sera that react slowly with antigen and have flat neutralization curves and a high degree of dissociation from antigen *Jerne* (10) expressed an increase in avidity by increasing values

of the association constant Since the term "avidity" has been used to include both equilibrium and rate constants we have preferred to discuss the present results in terms of differences in the affinity of antibodies

It has long been known that the affinity of antibodies increases during the course of immunization (10–14) The most illuminating work pertaining to this aspect of the immune response is contained in *Jerne's* studies of the diphtheria toxin-antitoxin reaction (10) and the neutralization of bacteriophage T4 (12, 17). *Jerne* (10) reported that early antitoxic sera were of lower combining power than sera from later bleedings and that the neutralization of T4 by early serum was reversible on dilution (12) Another important contribution in this field is the study by *Eisen & Siskind* (18) of the affinities of anti-2,4-dinitrophenyl antibodies for different dinitrobenzenes These authors reported that the average intrinsic association constant, measured by fluorescence quenching, increased markedly with time after immunization

The possibility that antibodies of different affinity are directed against different antigenic groups on the PV particle must be considered Results from immuno-diffusion tests (8) were compatible with, but did not prove, that 19S and 7S antibodies of different affinity reacted with the same antigenic component of the virus We may recall, however, that *Landsteiner & van der Scheer* (19) studying the affinity of rabbit antibodies against the single determinant group m-aminobenzenesulphonic acid concluded that the antibody was heterogeneous in affinity for this single determinant group

Pauling (20, 21) was the first to attempt a quantitative analysis of the energetic heterogeneity of antibodies from measurements of hapten antibody interactions by equilibrium dialysis He proposed that the heterogeneity in antibody affinities could be described by the Gauss error distribution *Karush* (22), studying the combination of antibody and azohaptenic dyes by the same technique, suggested that the Gauss distribution could be used tentatively for the analysis of antibody complementarity The most simple alternative theory to Pauling's proposal would be the existence of only two types of antibodies of different combining strength, the molar ratios of which would determine the average affinity of a serum

This brings up the question of a possible relationship between the formation of 19S and 7S antibodies and the change in the affinity of antibodies following immunization In a recent study of the dissociation of complexes formed by bovine albumin (BSA) and anti-BSA antibody Grey (23) reported that 7S and 19S antibodies produced 7 days after immunization dissociated from BSA at similar rates This observation and the finding that 19S antibody formation is short-lasting (3, 23–26) suggested a lack of relation between 19S antibody formation and the change in antibody affinity The present results indicate that a concept of a discrete bimodal distribution as an explanation for the hetero-

geneity in antibody affinity would be an eversimplification of the actual situation Thus it was found that besides two main classes of 19S antibodies of different average combining strength there existed 7S antibodies of divergent affinity (Fig 8) The difference between 19S antibodies of low and high affinity was well illustrated by the finding that early 19S antibody-PV complexes were effectively dissociated by 10^{-5} M hydrogen ion while $10^{-3.0}$ to $10^{-3.4}$ M was required when the later appearing firmly combining 19S antibody was employed A serum can therefore contain one or several antibody populations of differing affinity, each one of which may or may not conform to a normal distribution Only in extremely early or in late antisera was antibody of fairly uniform affinity found It is of interest that when the Gauss distribution was considered to describe adequately the heterogeneity in antibody affinity (21, 22) equilibrium dialysis or light-scattering methods which require hyperimmune sera were used In contrast, the ammonium sulfate precipitation technique by Farr (13), which also allows a study of the antigen binding capacity of primary response sera suggested a greater heterogeneity in antibody "avidity" in a primary than in a secondary response serum (16)

Differences in net electrical charge between antibodies with different specificity or between L-chains from such antibodies have been reported (27) Further, the electrophoretic mobility of the S-fragments, which contain the antibody combining site has been closely related to

... related to the same structural change in antibody molecules

The firmness of PV-antibody complexes was tested by exposing such complexes to hypertonic sodium chloride solutions (29) This technique, as in the case of acid-dissociation, revealed marked differences in the dissociability of complexes formed by PV and antibodies produced at different stages of the immunization process When 4- or 5 day IRS were used an increase in ionic strength of the virus serum mixture caused a rapid virus reactivation which was proportional to the ... tonicity ...

IRS ...

sera ... prior to testing

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The inability to demonstrate 19S γ_1 globulin antibody of high affinity during the first 2 ½ days after the intravenous injection of a high dose of PV (7×10^8 to 3×10^9 PFU) may be due to the presence of excess antigen in the circulation which would combine preferentially with antibody of high affinity and thus rapidly remove this antibody The clearance time for 3×10^9 PFU of PV varied between 20 and 36 hours

An alternative explanation for the change from weakly combining 19S γ_1-β globulin antibodies to 19S γ_1-globulin antibodies of high affinity would be that it is associated with the process of cell maturation This interpretation is compatible with the reappearance of 19S antibody of low affinity (recruitment of immature cells) following restimulation with PV and with the kinetics of the cytological changes which occur in the rat (30) and rabbit spleen (31) following injection of antigen

No weakly combining 7S antibody to PV was demonstrable while both normal and early immune 19S antibodies of low affinity were produced The 7S antibody in late IRS formed highly stable complexes with the viral antigen It is tempting to speculate about the possible significance of these findings in relation to two important differences between the formation of 19S and 7S antibodies to PV, namely the distinctly lower antigen dose requirement for induction of 19S antibodies and the more efficient retention of immunological memory in 7S formation (3, 8, 25)

SUMMARY

The dissociation of complexes formed by poliovirus and neutralizing antibody of rabbit origin was studied at increasing hydrogen ion concentrations ($10^{-6.5}$ to $10^{-2.5}$ M) This technique revealed the presence of two 19S antibody populations of distinctly different combining strength as well as 7S antibodies of different affinity 19S antibodies of low and high affinity were associated with γ_1-β- and γ_1-globulins respectively Firmly binding 19S and 7S γ_1 globulin antibodies produced early in the course of immunization were of similar combining strength The average affinity of the antibody in a particular serum appeared to be determined by the molar ratios between three principal classes of antibodies in regard to combining strength, namely, 19S γ_1-β globulin antibody of low affinity, more firmly combining 19S γ_1-globulin antibody or 7S antibody and 7S γ_2-globulin antibody of high affinity

About 10 per cent of rabbit sera contained normal 19S γ_1-β globulin antibodies of low affinity The sequence of the appearance of circulating immune antibodies of different affinity following a single antigenic stimulation were during the first few days 19S γ_1-β-globulin antibodies of low affinity, beginning on day 3 firmly combining 19S γ_1-globulin antibodies, from days 4 or 5 firmly combining 7S γ_1-globulin antibodies and from 2 weeks on 7S γ_2 globulin antibodies of high affinity Restimulation with antigen resulted in a reappearance of 19S antibodies of low affinity

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STUDIES ON THE PHENOTYPIC EXPRESSION OF COMPETENCE IN *NEISSERIA MENINGITIDIS*

By

SVERRE LIE

Received 24 x 64

In bacterial transformation, competence has been defined as the capacity of bacteria to absorb DNA molecules from their environment and to become genetically transformed (22) The exact nature of competence is unknown, but in most species studied it seems to develop only during certain specific growth periods In the three species most thoroughly examined *Pneumococcus* (21), *H influenzae* (19) and *B subtilis* (2), competence develops in the later part of the exponential growth phase and the early stationary phase In *Pneumococcus* the rise and fall of competence of a culture is especially abrupt with the individual cell remaining competent for 15 minutes or less (21, 9) There is some recent evidence that the explosive occurrence of competence in this species is associated with the production of some macromolecular substance which can confer competence on otherwise incompetent cells (22) In the same species the actual state of competence has been associated with some sort of unbalanced growth (6)

In an earlier publication from this laboratory, the genetic basis of competence in *N meningitidis* has been studied (12) Evidence was presented to indicate that the ability to be transformed by DNA is determined by the presence of a genetic determinant that probably is of the plasmid type

The present paper is concerned with the phenotypic variation in competence of meningococcal cells genetically capable of being transformed by DNA The results obtained have been compared to those of other well known transformation systems to see whether any principal differences could be detected

MATERIALS AND METHODS

Bacterial strains A streptomycin sensitive, histidine requiring competent [illegible] (mutant 12 str s his [illegible]) was [illegible] as isolated [illegible] ant of the recipient strain resisting more [illegible] µg streptomycin per ml (14)

25 *Uhr J W & Finkelstein M S* Antibody formation IV Formation of rapidly and slowly sedimenting antibodies and immunological memory to bacteriophage ØX 174 J Exp Med *117* 457 1963
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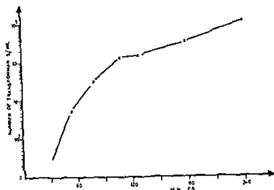


Fig 1

Kinetics of phenotypic expression of streptomycin resistant transformants Zero time = DNase addition to a culture exposed for 30 minutes to str r DNA

In some experiments the phenotypic expression was followed in liquid medium incubated without shaking It was found that this deeply influenced the kinetics of appearance of transformants The time required was much longer (on the average 150–180 minutes) and the appearance followed a much more irregular pattern Good growth conditions is therefore needed to get the optimal picture of phenotypic expression

In all the transformation experiments to be described, the expression of transformants has taken place on complete agar plates From Fig 1 one might expect that an incubation period of 3 hours would be sufficient for the expression of all transformed cells However, a period of 5 6 hours was found to be the expression time actually needed This discrepancy is most probably due to the fact that meningococci are very sensitive to changes in environment and that the transfer from liquid to solid medium will lead to a definite lag in the onset of division of the meningococcal cell

2 Competence as Related to the Growth Cycle

In *Pneumococcus H influenzae, B subtilis* and *Streptococcus*, cells with the potential capacity of becoming transformed develop this property only during a specific period of the growth cycle (9, 21, 1, 19, 2, 16) In order to determine whether such periods of competence existed in meningococci an overnight culture was diluted 1 200 in fresh BH and incubated with shaking at 37° C (starting titre around 1×10^7 cells per ml) Growth was followed both by measuring the increase in turbidity and viable count At various points on the growth curve the cells were tested as recipients in transformation using 20 μg str-r DNA per ml, and the general transformation technique described In Fig 2 are presented the results of one typical series of experiments In contrast

General transformation procedure In all the transformation experiments to be described, sensitive cells were transformed to streptomycin resistance Recipient cells grown in brain heart infusion broth (BH) to the desired growth phase were diluted 1:10 in prewarmed BH containing the desired amount of str r DNA and 0 005 M $CaCl_2$ The reaction mixture was incubated in a 37° C waterbath for the desired period of time (usually 30 minutes if not otherwise stated), and the reaction ended by adding DNase to a final concentration of 50 μg per ml After another five minutes of incubation to ensure the destruction of all DNA not irreversibly bound to the cells, the cells were spread on the surface of complete agar plates (usually blood agar) and incubated for phenotypic expression of the transformed marker After growth for about 5 6 hours the agar was moved to another of the same composition and volume containing 200 μg streptomycin per ml A detailed description of this technique has recently been given by *Bovre* (4) Counting of transformant colonies could usually be done after 24–30 hours of incubation

Controls included parallel transformations with DNA pretreated with DNase for 5 minutes Transformation frequencies (T Γ) are expressed as the number of transformants (T) per exposed cell (Γ)

RESULTS

1 *The Kinetics of Phenotypic Expression of Transformed Cells*

Catlin (5) found that phenotypic expression of transformed markers in meningococci mainly takes place during the 80–140 min period after DNase addition, and that newly expressed transformants still appeared at a time when the first to be expressed already had started to divide This finding is of great importance as it renders it impossible to make quantitative transformation work with phenotypic expression in liquid media In *Pneumococcus,* on the other hand, phenotypic expression is completed approximately 90 minutes after DNase addition and these expressed transformants show a lag of more than 1 hour before they start dividing again (10) To examine the kinetics in our system, cells exposed to DNA for 30 minutes with subsequent DNase treatment were diluted 1 10 in prewarmed BH and incubated with shaking at 37° C Another aliquot was diluted and spread on a number of blood agar plates and incubated At various intervals, the number of transformants in the liquid culture was determined by spreading 0 1 ml of various dilutions on complete agar plates containing 100 μg streptomycin per ml Blood agar plates were removed to plates containing 200 μg streptomycin per ml after 2, 4, 5, 6, and 7 hours of incubation The phenotypic expression in liquid medium is presented in Fig 1 The number of transformants increases rapidly from 30 120 minutes after DNase addition At 120 minutes there is a small tendency towards a plateau, but the second increase starts soon afterwards The number of transformants obtained at 120 minutes of incubation agrees well with that obtained on blood agar plates The second increase is therefore considered to be due to the division of already expressed transformants It can be concluded therefore, that while expression is virtually completed within 120 minutes of incubation, the secondary rise due to growth of the transformants takes place too early to justify expression in liquid medium in quantitative transformation work

of transformants obtained in the experiment reported in Fig 2 is considered insignificant In many other series of experiments such small variations were not observed Catlin (5) has observed a similar distribution of competence in a growing population of meningococci in liquid medium

The results shown in Fig 2 are those to be expected if unsaturating concentrations of transforming DNA had been used To exclude this possibility titration curves were made of all the DNA preparations used in such experiments One typical titration curve is presented in Fig 3 It is seen that below a certain concentration of DNA the number of transformants obtained is directly related to the concentration of DNA This indicates that one molecule of DNA is sufficient for a transformation to take place A point of saturation is reached however beyond which any further increase in the concentration fails to increase the number of transformants obtained This type of DNA titration curve has been obtained in a number of species (1 17 10 2) In all the experiments of the type reported in Fig 1 the concentration of DNA used was well above the saturation level found in the titration curve

The advantage of preserving cells in the state of competence for longer periods of time has been achieved in *Pneumococcus* and *H influenzae* by the addition of 10 15 per cent glycerol with subsequent freezing of the culture (7 8) To see if the same could be achieved in *meningococci* cells were frozen with and without glycerol in the early and late exponential growth phase and in the stationary phase It turned out however that the cells were very sensitive to freezing and the titre of the suspensions regularly decreased by a factor of around 10^3 every 24th hour Cells for use as recipients in transformation experiments therefore had to be prepared on the day of use Measurements of the optical densities were regularly used as a reference in order to get the populations in the best and most comparable states of competence (Fig 2 From day to day however there was regularly observed great fluctuations in the transformation frequencies obtained in identical experiments

3 *Kinetics of DNA Absorption*

In the experiments reported so far the time of exposure to DNA has been 30 minutes The relationship between the exposure time and the yield of transformants was investigated by inoculating 0 5 ml of recipient culture into 4 5 ml of prewarmed BH containing 0 005 M CaCl and -0 µg str r DNA per ml The mixture was incubated at 37° C with slow gyratory shaking and samples removed at various times and mixed with DNase to stop any further reaction Transformants were scored on blood agar plates by the usual technique In Fig 4 is reported the result of one such experiment Up to 30 minutes of exposure there is almost a linear relationship between the exposure time and the number of trans

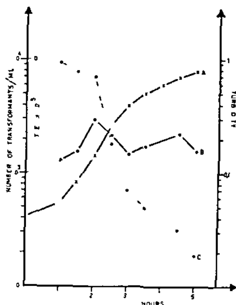


Fig 2

Competence related to the growth cycle of *N meningitidis*

— × — × — × — × — = Growth curve of the recipient cells (right ordinate)
— ● — ● — ● — ● — = *Total number of transformants per ml (left ordinate)* Each point on the curve represents the time when samples were withdrawn from the growth flask and exposed to str-r DNA
-- o ---- o ---- o -- = Calculated transformation frequencies (T E) (left ordinate)

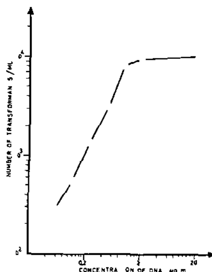


Fig 3

Influence of the concentration of str-r DNA on the number of transformants obtained

to other species used in transformation, the meningococci are seen to exhibit sensitivity to transformation throughout the entire growth cycle with the maximal transformation ratio being obtained in the early logarithmic growth phase The small variations in the absolute number

been exposed to str r DNA various times after washing It is seen that as soon as 10 minutes after washing most of the cells have lost competence No decrease in viable cell count could be observed during the first 60 minutes of incubation in saline

TABLE 1
Loss of Competence of Cells of N meningitidis when Washed and Resuspended in Saline

Period ran form e l after wa h ng m n)	Number of transforman s per ml	T E (× 10⁻⁵)
0 10	$1\ 1 \times 10^3$	13 75
5 15	$3\ 8 \times 10^2$	4 75
10-20	$3\ 5 \times 10^1$	0 44
15 25	10^1	0 1

Cells were grown h hak ng h

TABLE 2
Effect of Ca and Album n on the Transformat on in Sal ne of Washed Cells of N meningitidis

Transforma on med a	Number of trans forman s per ml	T E (× 10⁻⁵)
Sal ne S	8×10	2 7
S + 0 005 M Ca	$1\ 5 \times 10^4$	50 0
S + 0 5 % albumin	$1\ 6 \times 10^4$	53 3
S + 0 005 M Ca⁺⁺ and 0 5 % all min	$1\ 9 \times 10^4$	63 3

Th

During the first 10 minutes after washing however transformations could be performed in order to study the effect of various agents on competence *In Table 2* are presented the results of experiments in which the effects of Ca⁺⁺ and albumin have been studied Both of these two agents have been of importance in several transformation systems Already in the classical work of *Avery et al* (3) the requirement for albumin in H a was demonstrated and 6) Ca is stimulatory and *H influen ae* (8) the transformation of requirement for divalent cations is absolute (23) The latter authors postulate that this effect is due to a stabilization of the DNA molecule

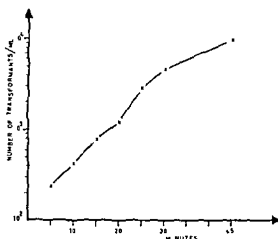


Fig 4
Rate of DNA absorption by competent cells of *N meningitidis*

formants obtained Exposure times longer than 45 minutes increased only slightly the number of transformants Under the conditions used it therefore seems to be a much slower absorption of DNA to the meningococcal cell than to the cells of *Pneumococcus* (9) and *H influenzae* (8, 20) Curves more camparable to that of Fig 3 have been found in *Moraxella* (4)

4 *Influence of Environmental Factors*

The transformations so far described have all taken place in complex media with Ca^{++}-ions added and with recipients grown with shaking If recipients were grown without shaking, the transformation frequencies were regularly lowered by a factor of 5 10 The same could be observed if the cells were preincubated in broth/saline mixture before transformation It is a general impression that the highest transformation frequencies were obtained in periods of maximal metabolic activities In *H influenzae*, for instance, maximal competence is obtained through three successive growth conditions Aerobic growth, anaerobic incubation and finally incubation in saline (20) In experiments where this procedure was employed with meningococci a progressive lowering of the transformation frequencies was obtained

To study the effect of various substances on the competence it is of great advantage to perform the transformations in defined media This could be achieved simply by taking the cells in the early exponential growth phase, washing them rapidly three times with saline on a membrane filter, resuspending them in saline and then perform the transformation immediately Very few cells had lost competence when this process was carried out at 37° C However, upon incubation in saline the cells very rapidly lost the capacity of becoming transformed In Table 1 is presented the results of experiments in which cells have

been exposed to str-r DNA various times after washing It is seen that as soon as 10 minutes after washing most of the cells have lost competence No decrease in viable cell count could be observed during the first 60 minutes of incubation in saline

TABLE 1

Loss of Competence of Cells of N meningitidis when Washed and Resuspended in Saline

Period transformed after washing (min)	Number of transformants per ml	T F ($\times 10^{-4}$)
0-10	1.1×10^{3}	13.75
5-15	3.8×10^{2}	1.75
10-20	3.5×10^{1}	0.44
15-25	10^{1}	0.1

Cells were grown by shaking to the early logarithmic growth phase washed rapidly [illegible]

TABLE 2

Effect of Ca^{++} and Albumin on the Transformation in Saline of Washed Cells of N meningitidis

Transformation media	Number of transformants per ml	T E ($\times 10^{-4}$)
Saline (S)	8×10^{2}	2.7
S + 0.005 M Ca^{++}	1.5×10^{4}	50.0
S + 0.5 % albumin	1.6×10^{4}	53.3
S + 0.005 M Ca^{++} and 0.5 % albumin	1.9×10^{4}	63.3

The preparation of recipient cells and the technique of transformation were as those described in the legend to Table 1 Exposure time was 10 minutes with subsequent growth for 7 hours on blood agar plates to allow phenotypic expression

During the first 10 minutes after washing however, transformations could be performed in order to study the effect of various agents on competence In Table 2 are presented the results of experiments in which the effects of Ca^{++} and albumin have been studied Both of these two agents have been of importance in several transformation systems Already in the classical work of Avery *et al* (3) the requirement for [illegible] ci was demonstrated and [illegible] (16) Ca^{++} is stimulatory [illegible] of both *Pneumococcus* (7) and *H influenzae* (8) and in the chemically defined medium used in the transformation of *B subtilis* the requirement for divalent cations is absolute (23) The latter authors postulate that this effect is due to a stabilization of the DNA molecule

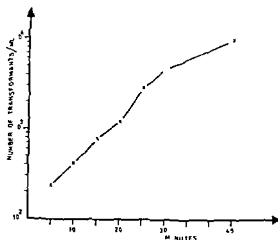


Fig 4
Rate of DNA absorption by competent cells of N *meningitidis*

formants obtained Exposure times longer than 45 minutes increased only slightly the number of transformants Under the conditions used it therefore seems to be a much slower absorption of DNA to the meningococcal cell than to the cells of *Pneumococcus* (9) and *H influenzae* (8, 20) Curves more camparable to that of Fig 3 have been found in *Moraxella* (4)

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TABLE 1

Loss of Competence of Cells of N meningitidis when Washed and Resuspended in Saline

Period transformed after washing (min)	Number of transformants per ml	T F (× 10⁻³)
0-10	1 1 × 10³	13 75
5-15	3 8 × 10²	4 75
10-20	3 5 × 10¹	0 44
15-25	10¹	0 1

Cells were grown by shaking to the early logarithmic growth phase, washed rapidly *two times* with saline on a Membrane filter (porosity 0 3 μ) and finally resuspended in saline to the original volume and incubated in a 37° C waterbath (time zero)

TABLE 2

Effect of Ca^{++} *and Albumin on the Transformation in Saline of Washed Cells of* N meningitidis

Transformation media	Number of transformants per ml	T E (× 10⁻³)
Saline (S)	8 × 10²	2 3
S + 0 005 M Ca^{++}	1 5 × 10⁴	50 0
S + 0 5 % albumin	1 6 × 10⁴	53.3
S + 0 005 M Ca^{++} *and 0 5 % albumin*	1 9 × 10⁴	63 3

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During the first 10 minutes after washing, however, transformations could be performed in order to study the effect of various agents on competence In Table 2 are presented the results of experiments in which the effects of Ca^{++} and albumin have been stud ed Both of ... [illegible]

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The results from Table 2 show that washed cells of meningococci have an almost absolute requirement for either Ca^{++} or albumin when transformation takes place in saline The effects of these two agents are only slightly additive, indicating that they might serve the same function In this connection it is of interest that is has been proposed that the effect of albumin may depend on some trace amount of substances complexed reversibly to the protein rather than to an effect of the protein *per se* (18) The results of Table 2 tend to support this conclusion, since it is more difficult to imagine that protein as such can serve the same function as a Ca^{++}-ion in promoting competence

The effect of Ca^{++} and albumin in complex media is less striking owing to the greater number of transformants obtained with transformations in media devoid of both agents However, the effect is readily detected as the results shown in Table 3 indicate

TABLE 3

Effect of Ca^{++} and Albumin on the Transformation in BH of Unwashed Cells of N meningitidis

Transformation media	Number of transformants per ml	T F ($\times 10^{-3}$)
BH	1.4×10^{3}	1 4
BH + 0 005 M Ca^{++}	8.5×10^{3}	8 5
BH + 0 5 % albumin	8.0×10^{3}	8 0
BH + 0 005 M Ca^{++} and 0 5 % albumin	1.1×10^{4}	11 0

Cells were grown to the middle of the logarithmic growth phase and diluted 1 10 in the transformation medium indicated in the table Saturating concentrations of str-r DNA were used with an exposure time of 30 minutes and with subsequent growth for 7 hours on blood agar plates to allow phenotypic expression

The frequency of transformation in meningococci under the conditions used is rarely greater than 0 1–0 2 per cent This frequency is lower than the ones obtained in most other systems and might indicate that the optimal conditions for transformation in this species have not been found That more than 90 per cent of the cells of a competent meningococcal culture is potentially capable of reacting with DNA could be demonstrated in the following type of experiment Blood agar plates were spread with 0 1 ml of undiluted str–r DNA solution and the plates afterwards spread with around 100 200 colony-forming units per plate The actual number inoculated was determined on a separate series of plates and controls included the inoculation of around 10^4 cells of the same population on blood agar plates devoid of DNA Growth was allowed to take place until microcolonies were visible to the naked eye, and the agar then moved to streptomycin-containing plates with the usual technique With such a procedure resistant transformants were found in the progeny of more than 90 per cent of the colony-forming units plated with str–r DNA It is concluded, therefore, that almost

every cell of the population carries the latent property of being transformed (12), but that at any given time only a small fraction has this property expressed In pneumococci, experiments principally similar to this has been performed with antigenic markers (17)

DISCUSSION

One of the most striking differences between the transformability of meningococci and that of most other species studied is the capacity of meningococci to transform throughout the entire growth phase The greatest transformation frequency (T E) is found in the beginning of the growth phase while most other species studied exhibit the greatest T E ratio in the late exponential and the early stationary phase

Several lines of evidence point to the hypothesis that competence in meningococci is directly related to the metabolic activity of the recipient population The highest T E ratio is found in the first part of the growth cycle, when there are superfluous nutrients and minimum of toxic substances Furthermore, in experiments where the metabolic activities of the cells were varied by incubating with and without shaking and by growth in broth diluted in saline, the highest transformation ratio was always obtained in the cultures with the shortest generation time Washing of the cells in saline did not affect the transformability of the population if transformation was performed immediately after washing However, the cells became completely incompetent within 10 minutes after washing, indicating that residual growth of the recipient cells was responsible for the transformation observed in saline

In most other species studied, cultures do not exhibit the maximal competence in periods with the most active metabolism, although the incorporation of DNA is an energy-requiring process (23) Reference has already been made to the finding that maximal competence of *Pneumococcus*, *H influenzae* and *B subtilis* is found in the latter part of the growth cycle and the beginning of the stationary phase (21, 19, 2) In *H influ*[illegible] three suc[illegible] of the ce[illegible] in saline [illegible] this procedure in meningococci only decreased the transformation frequencies obtained In *B subtilis*, *Nester* (15) has made some observations indicating that the competent cell is not growing and multiplying This hypothesis was in part based on the observation that competent cells are penicillin resistant, while incompetent were not Such differences have not been detected between competent and incompetent cells of meningococci (13)

One more finding that might be explained by assuming a high metabolic activity of the competent cell of meningococci is the kinetics of phenotypic expression of the transformed marker The principal difference between the type of expression illustrated in Fig 1 and the one

exhibited by pneumococci (10) is not the rate of phenotypic expression, but the rapidity with which the transformants start to divide after expression In pneumococci there is a period of 1–2 hours between expression and onset of division of the expressed transformant, while there is a period of only 5–10 minutes in meningococci This is in part to be expected if one assumes a great difference in the metabolic activities of the two cells at the time of DNA-uptake If competence in pneumococci is associated with low metabolic activity, the cell might well be able to express the transforming marker but it might not be able to start dividing again before a considerable period of time has elapsed *Ephrussi-Taylor* & *Freed* (6) have indicated that the competent cells of pneumococci are in some sort of unbalanced growth, and in *B subtilis*, *Nester* (15) has found that the competent cell actually remains in a non-dividing condition for as much as 3–4 hours If competence in meningococci, on the other hand, is associated with high metabolic activities, there is no *a priori* reason why the newly expressed transformants should not continue to divide almost immediately

SUMMARY

The phenotypic expression of competence in cells of *N meningitidis* has been studied It has been found that the cells are capable of being transformed all through the regular growth cycle The highest transformation frequency, however, is obtained in the early part of the logarithmic growth phase

Under various growth conditions, the cells are found to be most susceptible to transformation in media exhibiting the shortest generation time It has been proposed that in meningococci, in contrast to most other transformable species studied, the degree of competence of a culture is directly related to the metabolic activity of the cells

Gentle washing on a membrane filter did not decrease the competence of the cell population When transformation with such washed cells took place in saline there was an almost absolute requirement for either Ca^{++}-ions or albumin The effects of these two agents were not additive Washed cells resuspended in saline were found to loose the ability of becoming transformed within 10 minutes of incubation

Experiments are presented to demonstrate that almost all the cells of a competent meningococcal population are potentially capable of becoming transformed

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PRODUCTION OF RECOMBINANTS IN MIXED CULTURES OF *NEISSERIA MENINGITIDIS*

By

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Several bacterial species have been shown to release into the growth medium macromolecular products of the deoxyribonucleic type (DNA). Such species include for instance *Staphylococcus* (6), *Alcaligenes*, *Pseudomonas* and *Flavobacterium* (3), and *Micrococcus halodenitrificans* (22). Of special interest are the reports of *Catlin* (5) and *Ottolenghi & Hotchkiss* (15, 16) who studied the release of DNA in cultures of *N. meningitidis* and *Pneumococcus*, respectively. In these species, the presence of DNA can be detected by its ability to transform the appropriate competent cells (18). In meningococci, it was proposed that the production of DNA was a result of cell lysis. In *Pneumococcus*, on the other hand, the maximal amount of transforming activity was found before the culture had reached the stationary phase and coincided with the peak of competence observed in such cultures. There was, however, no real connection between the production of DNA and the ability to take up DNA (16).

In *N. meningitidis*, it has recently been observed that when two different auxotrophic mutants are plated together on a basal medium a high number of recombinants is obtained, provided at least one of the strains is able to be transformed by purified DNA (10). It was decided to study this phenomenon under more optimal growth conditions in order to get a better insight into the nature of the process observed. For this purpose, mutants resisting the action of either streptomycin or erythromycin have been selected and the production of double resistant types in mixed cultures studied. Furthermore, the kinetics of release of transforming DNA have been followed in liquid and solid complete medium. Finally, the system has been used in order to decide whether the ability to be transformed (competence) can be transferred from a competent to an incompetent strain.

I hereby wish to express my warmest thanks to Dr. K. *Jyssum* who introduced me to the field of microbial genetics and outlined the problems of the present studies. Without his continuous help and inspiring encouragement the present work could hardly have been accomplished.

MATERIALS AND METHODS

Bacterial strains In all experiments to be described, various mutants of strain 12,

tions a mutant resisting 25 μg erythromycin per ml was isolated and used in all experiments to be described This mutant strain was produced by at least 5 independent mutational events Incompetent variants of both mutants were obtained by spreading a culture for single colony isolations and testing a high number of such colonies for competence in transformation (11) Competent and incompetent strains will be designed as cp⁺ and cp, respectively

technique the bacteria are growth the entire agar is double concentrations of

3

the diffusion took place at room temperature to prevent any additional growth during the diffusion period

RESULTS

1 *The Formation of Recombinants in Mixed Culture*

In the classical paper of *Tatum & Lederberg* (23) bacterial conjuga-

The bacteria were grown to the middle of the logarithmic growth phase in BH, washed twice in saline on a membrane filter and 0 1 ml of each suspension added separately and in mixture onto blood agar plates After growth for about 4 hours, the agar was removed to another one of the same volume containing 200 μg streptomycin and 20 μg erythromycin per ml It is evident from Fig 1 that numerous recombinants resisting the action of both antibiotics are produced when the two mutant strains are allowed to grow together on a drug free medium for as little as 4 hours When such double-resistant recombinants were isolated, they exhibited a stable genotype with no tendency of reversion after several transfers on drug-free media

The production of recombinants could also be followed in liquid media Overnight cultures of the two strains were washed on a mem-

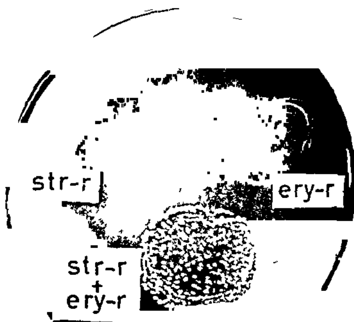


Fig 1

Production of recombinants on solid medium On a blood agar plate str-r cells and ery-r cells were added *separately* and *in* mixture After 4 hours of growth the cells were challenged to both antibiotics Numerous double-resistant recombinants appear *where the cells have been grown together.*

brane filter and 0 1 ml of each washed suspension added to 10 ml of BH and incubated with shaking at 37° C. Various time intervals after incubation aliquots were removed and plated undiluted on BH-agar containing 100 μg streptomycin and 10 μg erythromycin per ml Total growth of the culture was followed by optical measurements

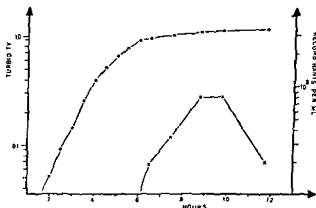


Fig 2

The appearance of recombinants in mixed, liquid cultures Str-r cells and ery-r cells have been added to 10 ml *BH* and incubated with shaking at 37° C The production of recombinants was followed by plating aliquots on media containing both antibiotics

Typical results from such an experiment have been presented in Fig 2 The recombinants do not appear until close to the stationary growth phase Maximum number of recombinants is observed around 3 hours after the culture has reached the stationary growth phase The decline thereafter is considered to represent the rapid inactivation of meningococci in stationary phase cultures

That such production of recombinants in mixed cultures is due to transformation of cells by DNA released from cells of the other genetic type was evidenced by the following findings The appearance of recombinants was completely prevented if DNase was added to the bacterial mixture at the time of plating Furthermore, if both parents were incompetent in transformation tests with purified DNA no recombinants could be detected Finally, if one competent strain was mixed with an incompetent, there was a unidirectional transfer of genetic material from the incompetent to the competent strain

This last finding was demonstrated by the following type of experiment Strain 12 cp⁺, ery–r was mixed with strain 12, cp , str–r in equal portions and 0 1 ml of the mixture plated on streptomycin plates (200 μg per ml), erythromycin plates (1 μg per ml) and on plates devoid of any antibiotic After 3–4 hours of growth the agar was moved to another of the same volume supplemented so that the final concentration of antibiotics after diffusion was 100 μg streptomycin and 0 5 μg erythromycin per ml In this type of experiment pregrowth on the drug free and on the erythromycin containing plates resulted in the production of numerous recombinants No recombinants were ever observed on the streptomycin containing plate Reverse experiments were also carried out in which the cp⁺ strain carried the str r allele The results were in principle the same with numerous recombinants except where pregrowth had taken place on erythromycin containing plates It was concluded that growth of the competent but not of the incompetent type is required for the production of recombinants which means that only the cp strain can act as a recipient of genetic material in such mixed cultures

It became apparent from such experiments that the number of transformants obtained when the competent strain carr[illegible] few, if any, recombinants appeared This is to be expected if the resistance level of the ery–r mutant used is a product of several independent mutational sites, each conferring a small degree of resistance and with no strong linkage in transformation tests It was considered beyond the scope of the present investigation, however to analyze the genetic basis of resistance to erythromycin in meningococci

2 *Production of Transforming DNA by Meningococcal Cultures*

It has been concluded that the recombinants which appear in mixed cultures of meningococci are produced by a process of transformation through the release of transforming DNA It was of interest, therefore, to analyse the kinetics of the release of such DNA by meningococci grown in liquid and on solid medium

a Production of DNA in liquid medium Cultures of str–r cells were started with an initial density of around 10^6 cells per ml and incubated with shaking at 37° C in BH Growth was followed by measuring the increase in optical density At various points on the growth curve samples were removed and placed in 4° C for subsequent analysis of DNA content Viability was not affected after 6 hours in the cold To detect biologically active DNA, 0 5 ml of donor culture was mixed with 0 4 ml of fresh BH and 0 1 ml of competent, ery–r cells $CaCl_2$ was added to a final concentration of 0 005 M (14) After around 30 minutes in a 37° C waterbath DNase was added to a final concentration of 10 µg per ml and the cells spread on complete agar plates After growth for 4–5 hours the cells were exposed to 100 µg streptomycin and 10 µg erythromycin per ml with the usual technique The results showed that no release of transforming DNA could be detected by this method during the regular growth cycle That the transformation mixture did not contain any substance(s) inhibiting transformation was shown by adding purified str–r DNA to some of the reaction mixtures The usual yield of transformants was then obtained After the culture had reached the stationary phase, however, transforming activities were found to accumulate in the medium This activity was mainly found in the cell-free filtrates of the culture and increased as the viable count of the culture decreased This DNA was very stable, and even after 35 days of shaking at 37° C a significant amount of DNA could be detected It is concluded, therefore, that in liquid medium no release of transforming DNA can be detected during active growth Furthermore, the source of the extracellular DNA found in the stationary phase is most probably dead cells

b Production of DNA on solid medium The transformants obtained in the type of experiment reported in Fig 1 might be obtained even before any visible growth of the parental cells can be observed This would indicate that during growth of meningococci the extracellular DNA does appear much earlier on solid than in liquid medium To investigate the time of appearance of such DNA, 0 1 ml of a suspension of washed str–r cells was spread on blood agar plates and incubated at 37° C After various hours of incubation, 0 1 ml of competent, ery–r cells were added, followed by the addition of excess amounts of DNase after another 40 minutes of incubation In order to get the most comparable results, the str–r cells were inoculated at different times to allow the use of recipient cells of the same level of competence In

control experiments DNase was added immediately before the recipient cells This completely prevented the formation of any recombinant After 4–5 hours of growth for phenotypic expression, the plates were moved to another of the same volume containing 200 μg streptomycin and 20 μg erythromycin per ml In Table 1 the results of one such experiment have been presented It is clear that DNA is released on the plates after only two hours of incubation and that the amount there after increases rapidly with time of growth

TABLE 1
Production of Transforming DNA during Growth of N meningitidis on Complete Medium

Time of growth before adding recipients	Number of transformants recovered
1 hour	2 × 10^1
2 hours	7 × 10^1
3 hours	1 2 × 10^2
4 hours	> 10^3

Streptomycin resistant cells were inoculated on a blood agar plate at zero time At the times indicated 0 1 ml of recipient erythromycin resistant cells was added, followed by DNase after 40 minutes of further incubation Phenotypic expression 4 hours selective medium 100 μg streptomycin and 10 μg erythromycin per ml

Such experiments do not yield any information about the absolute number of transforming units released from the cells during growth on the plate For instance, a number of transforming units might never get into an effective contact with the recipient cell In order to deter mine the absolute number of transforming units released, however, one must wash the bacterial growth from the plates and test it in liquid medium It was felt, that due to the fragility of meningococcal cells such experiments could not be performed without a significant interference by DNA released from cells ruptured by such manipula tions Furthermore the only purpose of the experiment was to demonstrate that DNA is produced during the early growth on solid medium rather than to obtain information about the absolute amount produced

3 *Infectivity of Competence*

In an earlier report from this laboratory the capability of being transformed was suggested to be determined by the presence of a genetic factor of the plasmid type (11) It was attempted to transfer competence to incompetent cells by mixed cultures on defined medium However, due to the lack of an appropriate selective system the results were inconclusive The present system offers a more sensitive method for the detection of a possible transfer of competence

Competent, str-r cells were mixed with incompetent, ery-r cells and

incubated on a blood agar plate containing saturating amounts of str r DNA After various hours of growth, the agar plates were moved to plates of the same volume containing 200 μg streptomycin and 20 μg erythromycin per ml This screening concentration of erythromycin is too high to allow the appearance of double-resistant transformants due to the transformation of str–r cells If transfer of competence did take place, however, the ery–r cells with this newly acquired property should be able to take up str–r DNA and segregate out the double resistant type In no instance could such a transfer be detected

The possibility remained, however, that such a transfer might occur only during a very small period of the growth cycle To elucidate this problem, the two mutant strains were allowed to grow together for 24 hours in BH The culture was then centrifuged, resuspended and diluted in fresh BH After growth to the logarithmic growth phase, str–r DNA was added and the cells spread on complete agar plates without DNase addition No recombinants were observed with a selective system of 100 μg streptomycin and 10 μg erythromycin per ml Under the present experimental conditions, therefore, it can be concluded that no transfer of the ability to become transformed takes place

DISCUSSION

In a previous report from this laboratory it was shown that recombinants are formed when two genetically distinct auxotrophic mutant of meningococci are plated together on a basal medium (10) This paper has demonstrated that recombinant types also appear on complete medium in high numbers when two different antibiotic-resistant mutants are allowed to grow together The following findings indicate that the cause of such recombinant formation is transformation 1 The production of recombinants is completely prevented if DNase is present in the system 2 At least one of the strains used must be competent in transformation when tested with purified DNA 3 When a competent cell is mixed with an incompetent, there is a unidirectional transfer of genetic material from the incompetent to the competent type

The production of recombinants takes place much earlier on solid than in liquid medium One possible cause of this difference could be the different selective techniques used Transformants produced in liquid medium are selected by direct plating on the drug-medium With growth on solid medium, the drugs must diffuse through the agar before the selective effect is obtained If there is a difference in the diffusion rates of the two antibiotics, the donor cells might be killed with subsequent lysis and release of DNA before the recipient cells are affected The use of drug-induced lysed cells as donor in transformation experiments have been described (8, 16) However, numerous recombinants were produced in mixed cultures of strains 12, cp⁺, ery r and 12, cp, str–r, in spite of the more rapid diffusion of erythromycin The release

of DNA with subsequent transformation must consequently have occurred before exposure to the drugs had taken place

In liquid medium no transforming activity can be detected until the culture has reached the stationary phase This would indicate that the source of transforming DNA is dead cells The earlier appearance of DNA on solid medium can be explained by the crowding of and competition between the cells within the microcolony formed after just a few generations The growth conditions within such a microcolony must be considered much less optimal than the one in liquid medium during active growth with a consequent earlier occurrence of cell death When recombination takes place on basal medium (10) growth for about 13 hours is required before DNase fails to prevent the formation of recombinants This is then most easily explained by the much slower growth on this medium

Catlin (5) was the first to describe the accumulation of extracellular, transforming DNA in old cultures of meningococci She did not, however report the production of recombinants in mixed cultures This has previously been observed in other transformable species such as pneumococci (15 16) and *B subtilis* (21)

There is no obvious *a priori* reason why the type of recombinant formation in mixed cultures of meningococci described in the present paper should not also exist *in vivo* This makes possible an adaptability of genetic material that is of great importance for the survival of this species under the most diverse environmental conditions For instance, the probability of the emergence of a mutant strain resistant to the action of two antibiotics is generally considered to be the product of the probabilities of mutating to either of the single-resistant types With the effective spread of a mutant gene through the population, however, the probability will obviously be much higher

With the accumulation of different transformable bacterial species in which recombination of genetic material takes place without experimental intervention it is reasonable to assume that there is in these species a much greater adaptability of genetic material than has hitherto been assumed and mutation can by no means be considered the only way by which new genotypes are produced Furthermore, the existance of transformation between closely related but not ident[illegible]
spe[illegible]
poo[illegible]
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tior[illegible] transformation processes of two or more properties such as virulence and drug resistance, or two biochemical capacities within one strain might appear to give almost *de novo* production of a new pathogenic species capable of invading effectively a new host or medium"

The ultimate requirement for transformation is of course that the cells are in a competent state In meningococci this capacity has been

suggested to be determined by the presence of a genetic determinant probably of the plasmid type (11) The system of recombinant formation in mixed cultures has been used to demonstrate the possible transfer of such a factor from a competent to an incompetent strain The results were altogether negative and it was concluded that under the experimental conditions used no transfer did take place However, incompetence might be due to one of two phenomena Either the cell does not absorb the DNA-molecule, or, if it is absorbed, it fails to intergrate and/or express the transforming marker A positive result of the experiments performed would mean that the incompetent cell actually was able to absorb the hypothetical factor A negative result, on the other hand, might either mean that the factor does not exist or that the cells are incapable of taking it up That the state of incompetence indeed is correlated with the ability of the cells to absorb DNA is indicated in the transformation systems of pneumococci (12), *H influenzae* (7), and *B subtilis* (24) Furthermore, in the few instances where mutant genes have been shown to affect transformability (24 19), the incompetent state is always followed by a marked decrease in the uptake of DNA Even if the factor existed and could be released from the cell it must consequently be considered most unlikely that it could be transferred to an incompetent cell

SUMMARY

The production of recombinants in mixed cultures of *N meningitidis* has been studied With the two strains carrying respectively the resistance markers to streptomycin and erythromycin, a high number of double resistant recombinants was produced during mixed growth of the two strains Evidence indicated that the cause of this recombinant formation was transformation through the release of biologically active DNA during growth

The production of transforming DNA was followed on solid and in liquid medium It is concluded that the source of this DNA is lysis of dead cells

The mixed culture technique was used to examine the possible transfer of the ability to be transformed from a competent to an incompetent strain No such transfer could be detected

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TRANSACTIONS OF THE MEDICAL MICROBIOLOGY DIVISION OF THE SWEDISH MEDICAL SOCIETY

Annual Meeting November 27–28, 1964, Stockholm, Sweden

Virology

Bengtsson, S., Dinter, Z. & Philipson, L., Group of Cell Biology, and Institute of Virology, University of Uppsala, Uppsala, and the State Veterinary Medical Institute, Stockholm: ON THE MECHANISM OF PICORNAVIRUS INHIBITION BY GUANIDINE

The inhibitory effect of guanidine hydrochloride (GHCl) on the multiplication of foot-and-mouth disease virus is suppressed by lactalbumin hydrolysate and to a certain extent, by Eagle's minimal essential medium This effect is due to some amino acids, mainly to methionine but also to leucine, valine and threonine The optimal concentration of methionine is 0 5 to 1 mM These amino acids also suppress the inhibitory action of GHCl on the plaque forming capacity of poliovirus type 1 in HeLa cells, but choline was found to have a stronger antagonistic effect A systematic study of substances structurally similar to choline showed dimethylethanolamine and dimethylpropanolamine to be even more effective; these substances gave a recovery of about 80 per cent of the virus present in the control when used at a concentration of 0 05 mM with 0 5 mM GHCl Multiplication studies show a lag of at least 12 hours before synthesis starts in the presence of dimethylethanolamine and GHCl It is tentatively suggested that the effect of GHCl is due to interference with some amine or aminelike substance necessary for synthesis of the virion

Marquardt, J., Holm S F & Lycke E, The Municipal Virological Laboratory, Gothenburg, and Department of Bacteriology University of Gothenburg, Gothenburg STUDIES OF THE ANTIGENIC STRUCTURE OF VACCINIA VIRUS

Infection of cells with vaccinia virus will give rise to the formation of a number of soluble antigens By means of immunoprecipitation analysis the precipitinogens found in materials of soluble antigens corresponded immunologically to precipitinogenic factors released from materials of purified virus particles

A number of precipitinogens were released from the outer protein layer of the vaccinia virus particle by digestion with pepsin One of these factors reacted immunologically as the LS antigen of vaccinia The haemagglutinin of vaccinia virus showed no precipitinogenic activity

Öberg B Albertsson P A & Philipson L Group of Cell Biology and Institute of Biochemistry University of Uppsala Uppsala COUNTER CURRENT DISTRIBUTION OF POLIOVIRUS RNA

Partition coefficients for some polynucleotides and virus RNA as well as intact poliovirus have been determined in an aqueous polymer two phase system with different ionic compositions (Dextran 500 5 per cent w/w—Polyethylen glycol 6000 4 per cent w/w) Counter-current distribution of poliovirus poliovirus RNA and TMV RNA has been performed in these phase systems A new type of apparatus for counter-current distribution was used in these model experiments The distribution curve for poliovirus RNA is in good agreement with the theoretical distribution and 10–60 per cent of the infectivity is recovered Intact poliovirus and poliovirus RNA are well separated with 60 transfers This number of transfers also gives a minor separation of polivirus RNA and TMV RNA

Holme T Department of Bacteriology Karolinska Institutet Stockholm ANTIGENS OF PHAGE RESISTANT MUTANTS OF A STRAIN OF SALMONELLA TYPHIMURIUM

The specific O antigen governs the effectiveness of Salmonella vaccines This antigen constitutes part of the endotoxin that is located in the cell wall of the bacteria In earlier studies of virulence and immunogenicity of *Salmonella typhimurium* auxotrophic mutants were employed with the aim of obtaining a vaccine with low toxicity but with retained immunizing potency Since the immunogenicity depends on components of the cell wall it appeared that the use of mutants selected for some difference in the properties of the cell wall would constitute a better approach Mutants with different cell wall characteristics may be selected with the aid of bacteriophages Receptors for these reside in the O antigen and phage resistant mutants can be expected to display a different O antigen

In the present investigation a series of phage resistant mutants of a strain of *Salmonella typhimurium* were selected with the aid of different phages of the phage typing set of Lilleengen and with Felix anti O phage The mutants were analysed with respect to virulence and immunogenicity for mice, antigen analysis was made by the agar gel precipitin test and agglutination and the sugar components of the antigenic determinants were analysed by thin film chromatography

Some of the phage resistant mutants fully retained their virulence and immunogenicity no differences between them and the parent strain in serological or chemical characteristics could be detected by the methods used Lipopolysaccharide from the cell wall of mutants with lost virulence and immunogenicity lacked certain sugars characteristic for the parent strain namely abequose and rhamnose A spontaneous R mutant derived from the same parent strain lacked mannose in addition The gel precipitin test revealed distinct differences between the different types of mutants

Rutberg B & Rutberg L Department of Bacteriology Karolinska Institutet Stockholm ON THE SUPERINFECTION IN LYSIS INHIBITION WITH BACTERIOPHAGE T4

Lysis inhibition with T even bacteriophage is characterized by a prolonged latent period and an increased burst size following superinfection with homologous phage of *E coli* primarily infected with wild type phage Some characteristics of the superinfection have been stud ed

The prolongation of the latent period is dependent on the time of superinfection whereas the increase in burst size is not Superinfecting phage inactivated with heavy doses of ionizing or non ionizing irradiation was able to induce lysis inhibition even at doses at which the killing property of the phage was lost Ghost particles of T4 were unable to induce lysis inhibition Lysis inhibited bacteria produce phage lysozyme at a slower rate than do non inhibited cells

It is suggested that upon superinfection some compound is injected into or produced in the bacteria and that this compound is consumed or otherwise made inactive during the continued synthesis of phage

Lycke E & Lund E the Municipal Virological Laboratory Gothenburg STUDIES ON THE METABOLISM OF TOXOPLASMA GONDII

The metabolism of toxoplasma was studied by means of a Cartesian micro diver method and a cell culture technique The respiration of extracellular parasites was determined to 1 to 2 × 10^{-7} µl/hour and parasite The toxoplasma infection of HeLa cells caused an increased respiration which seemed to be due to an increase of the oxygen consumption Parasites which devided in spite of treatment had a mide treatment of parasites inhibited the multiplication but did not affect the respiration while treatment with actinomycin D inhibited the growth and increased the oxygen consumption Parasites which divided in spite of treatment had a generaton time which was the same as the one found for untreated control parasites The compounds studied with capacity to inhibit the multiplication did not alter the ability of toxoplasma to infect *i e* penetrate HeLa cells

Strannegard Ö Lycke E & Lund E the Municipal Virological Laboratory Gothenburg THE EFFECT OF ANTIBODIES ON THE INFECTIVITY OF TOXOPLASMA PARASITES STUDIED BY MEANS OF A CELL CULTURE METHOD

The effect of antibodies on *Toxoplasma gondii* can be determined with a cell culture method where the capacity of the parasites to penetrate into HeLa cells is measured In Sabin Feldman s dye test antibodies require the participation of activator factors (the properdin system) to exert a demonstrable effect In the cell culture it was found that each of the activator factors enhanced the action of antibody but were possibly with the exception of properdin not absolutely necessary for the inactivation of the parasites A penetration inhibiting effect was found in activator sera without any demonstrable titre of dye test antibodies This effect was probably due to the action of the properdin system in combination with low levels of antibodies which were not detectable in the dye test The activator factors were not absorbed to the parasites in absence of specific antibodies 7S and 19S antibodies against Toxoplasma both appeared to require the participation of the activator system for their action

It seems as if properdin plays an essential role for the inactivation of Toxoplasma The findings are in some respects incompatible with the view that properdin represents a group of normal cross reactive antibodies

Immunology

Lundholm L Department of Bacteriology, University of Gothenburg Gothenburg

STUDIES ON A PRIMARY ANTIBODY RESPONSE INITIATED IN VITRO

A new system for the study of the primary antibody response initiated in vitro is described The fluorescent antibody technique has been applied to demonstrate the development of antibody synthetizing cells

Cells from the adult rat spleen are cultured on a monolayer of rat embryonal cells The medium consisted of Parker 199 supplemented with foetal calf serum at a ratio of 70 30 (v/v) The antigen diphtheria toxoid or tetanus toxoid, was introduced into the medium on the first day of culture of the spleen cells The dosage of antigen has been varied between 0 1 and 8 flocculating units per 2 ml of medium

At varying times the cultures were versenated and smears prepared On the third and fourth day but not earlier or later a few cells were seen to transmit pyroninophile material to other cells through intercellular bridges

The first antibody synthetizing cells were detected on the fifth day of culture On the seventh and eighth day antibody synthetizing cells were detected to have a clonal distribution

Varying blocking tests were included as controls and crosslabelled experiments have also been performed

Svehag S E Institute for Virus Research Karolinska Institutet Stockholm

SECONDARY RESPONSE OF 19S AND 7S ANTIBODIES TO POLIOVIRUS IN *VITRO*

Membrane cultures of separated spleen cells from rabbits injected once with poliovirus formed antibody *in vitro* for 2–3 weeks Restimulation of spleen cells in suspension with homologous antigen evoked secondary antibody responses The secondary 19S antibody response was rapid and transient The secondary 7S response which appeared to be biphasic, was of longer duration and reached peak titres about 12–13 days after antigenic restimulation Antibody production was prolonged when spleen cells were cultured together with a feeder layer of rabbit embryo fibroblasts Incorporation into the medium of chloramphenicol (50 μg/ml) or actinomycin D (5 μg/ml) inhibited the continued primary 19S antibody synthesis

Högman C F Johansson S G O & Killander J Blood Transfusion Service and Department of Clinical Chemistry, University Hospital Uppsala QUANTITATIVE ESTIMATION OF IMMUNOGLOBULINS ON ANTIBODY COATED RED CELLS

Most of the results reported in this communication were presented at the 10th Congress of the International Society of Blood Transfusion and will be published in the Proceedings of this Congress

Jonas n J Espmark A & Fagraeus A National Bacteriological Laboratory Solna

PREPARATION OF AN ANTI HUMAN SERUM BY IMMUNIZING RABBITS WITH MONKEY GLOBULIN

Antiglobulin sera prepared by immunizing rabbits with ammonium sulphate precipitated serum globulin from cynomolgus monkeys reacted with human antibody globulin The primary injection of cynomolgus globulin was given to the rabbits intramuscularly in Freunds complete adjuvant If the rabbits were boo

stered intravenously one month later sera obtained ten days after the booster injection were found to contain antibodies to cynomolgus γM globulin but no or very small amounts of antibodies to cynomolgus γG globulin If the rabbits were boostered once more after another month the sera obtained 10 days afterwards contained anti γG as well as anti γM

The sera obtained after the first booster injection were found to crossreact with human γM globulin and the sera obtained after the second booster injection cross reacted both with human γM globulin and human γG globulin The sera obtained after the first booster injection agglutinated sheep red cells sensitized by a sub agglutinable dose of human anti sheep red cell serum the anti red cell antibodies of which in 90 per cent were of the γM class (This serum was kindly supplied by dr Aho National Serum Institute Helsinki Finland) Agglutination was obtained up to the serum dilution 1/2000 Human Rh(+) red cells sensitized by incomplete anti Rh were not agglutinated

The anti cynomolgussera obtained after the first booster could be utilized to distinguish between γG and γM human anti thyroglobulin by the mixed haem adsorption technique Two fold dilution series of γG and γM fractions respectively of human anti thyroglobulin obtained by separation on a Sephadex G 200 column were tested against human thyroglobulin according to a mixed haemadsorption procedure applied to soluble antigens With indicator cells coated with an exterior layer of anti human globulin both γG and γM anti thyroglobulin was traced and this result was independent of whether the cells had an interior coating of human or of cynomolgus anti sheep red cell antibody When using indicator cells coated with an exterior layer of anti cynomolgus globulin only γM anti thyroglobulin was traced if the cells had an interior layer of human anti sheep red cell antibody and none of the anti thyroglobulin classes was traced if the interior layer consisted of cynomolgus anti sheep red cell antibody

Nystrom B The Central Laboratory for Clinical Bacteriology, Karolinska Sjukhuset Stockholm INFLUENCE ON BETAHAEMOLYTIC STREPTOCOCCI OF SERUM FROM PATIENTS WITH RHEUMATOID ARTHRITIS

The phagocytosis process in leucocytes from patients with rheumatoid arthritis and from healthy persons was compared The technique used was that of *Cohn & Morse* (J exp med 1959 110 419)

No differences were found between the phagocytosis of Staph albus in leucocytes from healthy persons and from patients with rheumatoid arthritis

With betahaemolytic streptococci of group C however highly significant differences were found in the phagocytosis after 30 minutes While the numbers of bacteria both totally extracellularly and intracellularly increased after 30 minutes in healthy persons it remained unchanged or decreased in patients with rheumatoid arthritis

This seems to be explained by a serum factor in rheumatoid arthritis influencing the streptococci It was not possible to wash this factor away from the leucocytes with ordinary washing procedures The growth of streptococci in rheumatoid arthritis serum was significantly suppressed compared to the growth in normal human serum This difference disappeared in heat inactivated serum No difference was found between the growth curves of Staph albus in untreated serum from patients with rheumatoid arthritis

Serum from patients with rheumatoid arthritis had no influence on the chain length of the streptococci as studied with the long chain test of Stollerman

Fjällbrant B Institute of Bacteriology University of Gothenburg and the Second Department of Obstetrics and Gynecology, Sahlgrenska Sjukhuset Gothenburg

IMMUNOAGGLUTINATION OF SPERM IN CASES OF STERILITY

A report is given of eleven cases of immunoagglutination of sperm Nine of them were found in a sterility material comprising 263 sterile marriages In all the eleven cases the male partners were healthy and had intact genital organs Ten showed normal sperm density only one a slight oligospermia The antibodies against sperm were revealed because of heavy spontaneous agglutination of sperm which was apparent both macro and microscopically The agglutinin titres and the sperm immobilizing effect of the men s blood sera has been determined Common to all the cases is a reduced ability of the spermatozoa to invade ovulatory cervical mucus both on slides and at postcoital tests Several facts strongly suggest that the immunoagglutination of sperm is the main cause of sterility in these cases It is concluded that heavy agglutination of sperm noticed at the investigation of sterile marriages should cause serological testing of the male partner for antibodies against sperm A film which demonstrated the diagnosis of autoimmunisation to sperm was shown

Navalkar R Department of Bacteriology, University of Gothenburg Gothenburg

IMMUNO DIFFUSION ANALYSES OF LEPROSY SERA

The work reported is a continuation of previous studies on detection and identification of precipitating antibodies in sera of leprosy cases by the use of a double diffusion plate method employing mycobacterial antigens and their corresponding antisera

In the present investigation two methods were used Method 1—a direct test with only antigen and Method II—a test referring to a so called reference system—an antigen and its corresponding antiserum The antigens used were Lepromin BCG protoplasm and concentrated culture filtrates of *M kansasii M smegmatis M avium M fortuitum M marianum M balnei M tuberculosis* and *M bovis* var BCG Among these antigens M kansasii was best suited for detecting precipitins in the leprosy sera Method II was especially suitable for revealing precipitins at low concentration Two different precipitins were identified—anti beta corresponding to the antigenic factor beta common to most of the mycobacterial species and anti-delta corresponding to the antigenic factor delta which is found in only a few of the species

Sixty sera from cases belonging to the three types of leprosy *i e* Lepromatous Reactional Tuberculoid and Tuberculoid were studied Of the 28 sera from the Tuberculoid cases 21 showed anti delta and only 2 showed anti beta Of the 17 sera from the Reactional Tuberculoid cases 15 showed anti delta and 9 showed anti beta Of the 15 sera from the Lepromatous cases all showed anti delta and 12 showed anti beta None of the 30 sera from a control group gave a positive precipitin reaction

The detection of anti delta could be considered valuable from the diagnostic point of view due to the comparatively high frequency of this type of antibody in the majority of the sera from all the three clinical types The detection of anti beta could be of significance from the prognostic point of view since only 2 of the true Tuberculoid cases have shown the beta antibody as compared to the many cases that show the same antibody in the other two types

It is possible that the presence of anti beta and anti dela [illegible] ue to some other mycoba[illegible] two anti bodies in the [illegible] infection

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A new sampling unit with ampulla sealed transport substrate and hinged poly propylene sampling stick has been elaborated

b) testing of a selective chocolate agar substrate for gonococcal culturing Added to the substrate were 25 IU of polymyxin and 10 µg of ristocetin per ml When testing this substrate in parallel with ordinary substrate on more than 6 000 samples it was shown that there were 2 2 per cent more positive samples than when using only the conventional gonococcal chocolate agar In 1 8 per cent of the samples it was not possible to demonstrate gonococci on the selective substrate but only on the conventional substrate

c) the percentage of strains with reduced sensitivity towards penicillin (≥ 0 1 IU/ml) In 1963 this was about 19 per cent In the beginning of 1964 the percentage of strains with reduced sensitivity towards penicillin was found to be reduced from 17 per cent in January to 10 per cent in May During the autumn there was once again an increase with a peak of 22 per cent in August

Roepstorff S O Department of Bacteriology Municipal Hospital Sundsvall

EXPERIENCE WITH A SELECTIVE CULTURE MEDIA FOR GONOCOCCUS AND MENINGOCOCCUS

In the beginning of 1964 a selective culture media for *N gonorrhea* and *N meningitidis* was introduced by *Thayer & Martin*

This media contains 10 µg/ml of ristocetin which inhibits gram positive cocci and 25 µg/ml of polymyxin which inhibits gram negative rods

At the Bacteriology Department of the Hospital in Sundsvall the medium has been comparatively tested in routine diagnosis of gonococci and in detection of meningococci in nose throat and sputum

Parallel cultures were carried out on the traditional and selective media

Total inhibition of most of the normal flora in the throat rectum urethra and cervix was obtained while meningococci and gonococci were not or only partially inhibited The selective media gave an increase in isolation frequency of 13 per cent

No increase was observed in men In women 12 per cent gain was noted in cultures from urethra and cervix and more than 300 per cent from the rectum Persistence of gonococci after penicillin treatment was observed in 8 per cent which could not be detected by traditional media

Meningococcal carriers were detected 3 5 times more often with the selective media than the ordinary media Despite inadequate sampling the average carrier frequency was found to be 30 per cent of 224 tested soldiers and 10 per cent of ab ut 1000 civilians

During the investigation time 2 cases of epidemic meningitis occurred

Mentzing L O & Ringertz O National Bacteriological Laboratory Solna

SALMONELLOSIS IN INTERNATIONAL TOURISTS

In 1963 a total of 1309 cases of salmonella infection were recorded in Sweden 65 per cent of these were found to have been infected while visiting non-scandinavian countries.

Bacteriology

Wittboldt S & Kjellander J Bacteriological Laboratory, Karolinska Sjukhuset Stockholm SOME PRINCIPLES OF AN AUTOMATIC DATA PROCESSING SYSTEM FOR LABORATORIES

We discuss the principles of catching the first information and handling it in an optimal way

With an ADP system it seems possible to administrer the laboratory in a more rational manner and to give a better information service to the clinics

Kjellander J Ljunggren A & Wittboldt S Bacteriological Laboratory Karolinska Sjukhuset Stockholm APPLICATION OF A MACHINE DATA SYSTEM IN A BACTERIOLOGICAL ROUTINE LABORATORY

All information concerning the specimens and the results of culture and antibiotic sensitivity testing has been codified in the bacteriological routine by means of a simple mainly pneumonic code The material is transferred to punch cards and listed daily with an ordinary tabulating machine The administrative work has been simplified as compared to the manual system important statistics is easily obtained and epidemiological data has been compiled regularly

Automatically typed laboratory reports based on the information on the punch cards will be the next project tested

Gnarpe H & Edebo L Department of Bacteriology University of Uppsala Uppsala THE STABILITY OF LEUCOCYTES IN PROTEUS INFECTED URINE

During the last decades *B proteus* has frequently been isolated from the urine of patients with symptoms of urinary tract infections Although the bacteria have often been demonstrated in high concentrations the occurrence of leucocytes has been relatively sparse and irregular

This might be due to the fact that proteus produces large amounts of urease which decomposes the urea of the urine into ammonia and carbon dioxide resulting in an increase in pH to the pH range where stability of leucocytes is reduced It has been found that leucocytes are lysed at pH values above 8 0 in sterile buffers as well as in urine or broth made alkaline by proteus a result anticipated from the initial hypothesis

It remains however to be demonstrated that leucocytes are exudated and later on disintegrated during urinary tract infections with proteus

Alcaligenes faecalis and *Pseudomonas aeruginosa* have a similar but slower and less pronounced effect

Gastrin B & Kallings L O National Bacteriological Laboratory Solna GONORRHOEA PRESENT EPIDEMIOLOGICAL AND DIAGNOSTIC PROBLEMS

Against a background of an investigation into the frequency of gonorrhoea in Sweden in 1963 based on an annual material of about 100 000 samples examined at the National Bacteriological Laboratory in Stockholm (*Gastrin & Kallings* Acta Derm Venereol 44 286 294 1964) a report is given of

a) comparative studies between fresh transport substrate (according to Stuart) and the same substrate stored in nitrogen ampullae for up to 20 months The study was carried out with suspensions of freshly isolated gonococcal strains which were stored in the substrate for 1 24 and 48 hours at 22° C The number of surviving

bacteria were estimated by a colony count method The examinations were made after 3 5 9 and 20 months' storage of the transport substrate The results show that the ampulla substrate was just as good after 20 months' storage as the fresh substrate

A new sampling unit with ampulla sealed transport substrate and hinged polypropylene sampling stick has been elaborated

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With an ADP system it seems possible to administrer the laboratory in a more rational manner and to give a better information service to the clinics

Kjellander, J, Ljunggren A & Wittboldt S Bacteriological Laboratory, Karolinska Sjukhuset Stockholm APPLICATION OF A MACHINE DATA SYSTEM IN A BACTERIOLOGICAL ROUTINE LABORATORY

All information concerning the specimens and the results of culture and antibiotic sensitivity testing has been codified in the bacteriological routine by means of a simple mainly pneumonic code The material is transferred to punch cards and listed daily with an ordinary tabulating machine The administrative work has been simplified as compared to the manual system important statistics is easily obtained and epidemiological data has been compiled regularly

Automatically typed laboratory reports based on the information on the punch cards will be the next project tested

Gnarpe H & Edebo L Department of Bacteriology, University of Uppsala Uppsala THE STABILITY OF LEUCOCYTES IN PROTEUS INFECTED URINE

During the last decades *B proteus* has frequently been isolated from the urine of patients with symptoms of urinary tract infections Although the bacteria have often been demonstrated in high concentrations the occurrence of leucocytes has been relatively sparse and irregular

This might be due to the fact that proteus produces large amounts of urease which decomposes the urea of the urine into ammonia and carbon dioxide resulting in an increase in pH to the pH range where stability of leucocytes is reduced It has been found that leucocytes are lysed at pH values above 8 0 in sterile buffers as well as in urine or broth made alkaline by proteus a result anticipated from the initial hypothesis

It remains however to be demonstrated that leucocytes are exudated and later on disintegrated during urinary tract infections with proteus

Alcaligenes faecalis and *Pseudomonas aeruginosa* have a similar but slower and less pronounced effect

Gastrin B & Kallings L O National Bacteriological Laboratory Solna GONORRHOEA PRESENT EPIDEMIOLOGICAL AND DIAGNOSTIC PROBLEMS

Against a background of an investigation into the frequency of gonorrhoea in Sweden in 1963, based on an annual material of about 100 000 samples examined at the National Bacteriological Laboratory in Stockholm (*Gastrin & Kallings* Acta Derm Venereol 44 286–294 1964) a report is given of

a) comparative studies between fresh transport substrate (*according to Stuart*) and the same substrate stored in nitrogen ampullae for up to 20 months The study was carried out with suspensions of freshly isolated gonococcal strains which were stored in the substrate for 1 24 and 48 hours at 22° C The number of surviving

Of 632 urine samples from the same number of unselected gravidae at full term investigated in 1963–1964 38 contained 2 × 10⁵ or more bacteria per ml 30 (4 5 per cent) represented verified urinary tract infections

The distribution of O antigen groups in 308 *E coli* strains isolated from the urine of children with urinary tract infection in Gothenburg Sweden did not differ strikingly from that found in 183 *E coli* strains from the faeces of normal infants in Copenhagen Denmark (Ørskov)

Henning C Hambraeus A & Melen B National Bacteriological Laboratory Stockholm the Bacteriological Laboratory and the Medical Department for Renal Diseases S t Eriks Sjukhus Stockholm and the Bacteriological Laboratory Centrallasarettet Danderyd A CHEMICAL TEST FOR THE DETECTION OF URINARY TRACT INFECTIONS

The ability of actively metabolizing bacteria to reduce the colourless compound triphenyl tetrazolium chloride (TTC) to a red insoluble dye can be used as a clinical test for bacteriuria as showed by Simmons and Williams

The aim of this investigation was to determine the reliability of the TTC test for detection of significant bacteriuria (more than 100 000 bacteria per ml) under practical conditions

1144 urine specimens from in and outpatients at two hospitals were examined with quantitative and qualitative bacteriological methods and with the TTC test at three bacteriological laboratories

300 (85 per cent) of urine specimens which contained more than 100 000 bacteria per ml showed a positive TTC test Among 786 urine specimens with a bacterial count of less than 100 000 per ml 40 (5 per cent) gave a positive TTC test No simple reason was found to explain the discrepancy between the TTC test and the bacterial cultures

The usefulness of the TTC test as a screening method was discussed The importance of performing when possible standard bacteriological methods for detection of urinary tract infections is stressed

Lithander [illegible]
TH
BR [illegible]

Rabbits with experimental meningitis produced with different species of bacteria were given intravenous injections of benzyl penicillin or ampicillin

The concentration of these preparations in CSF was much higher in infected animals than in controls It increased with the inten [illegible]

[illegible] signs of meningitis had appeared Relatively high concentrations of benzyl penicillin were found in CSF following the injection of dead β streptococci into the cisterna magna despite the fact that the animals showed no signs of meningitis

The concentration of benzyl penicillin in brain was somewhat higher in rabbits with meningitis than in controls It was usually unrelated to the intensity of clinical signs of meningitis After injection of ampicillin in rabbits with Haemophilus influenzae meningitis however the preparation was found in brain almost exclusively in animals with intense signs of meningitis

During June–October 4548 tourists participating in conducted tours in which cases of salmonellosis were suspected have been studied Salmonella culture was carried out in 3963 cases and salmonella organisms were isolated in 511 cases The majority of these had been infected in Italy, Spain Greece or Rumania Many different salmonella serotypes were found some of them very rare in Sweden Thus 145 out of 148 registered cases of *S haifa* had been infected abroad

To study the protective effect of TAB vaccination and the prophylactic use of intestinal antiseptics a questionnaire was sent to 2870 of the travellers It was returned by 1867 As could be expected no difference in morbidity of salmonella enteritis was found between vaccinated and unvaccinated travellers A significant increase in morbidity however *was found among tourists regularly using in*testinal antiseptics The reason for this negative effect is not clear but it may be due to the effect on the normal intestinal flora

During 1963 the great importation of salmonella did not cause any large outbreak of salmonellosis within Sweden but it must be considered a serious problem calling for preventive measures

Hallander H O Department of Bacteriology University of Uppsala Uppsala

ROUTINE DIAGNOSIS OF ENTEROTOXIN B PRODUCING STAPHYLOCOCCI

Staphylococcal strains to be tested for production of enterotoxin B were grown on a solid medium described by *Casman* (Pbl Hlth Rep (Wash) 73 599 1958) enriched with 2 per cent proteinhydrolysate and covered with cellophane Incubation *was carried for 24 hours at 37° C This technique increased the production* of enterotoxin B 16 times compared to the yield in agitated liquid medium Gel diffusion was used for the detection of enterotoxin The antiserum was produced by immunization with an enterotoxin B fraction purified by gel filtration in Sephadex G 100 and subsequent electrophoresis on cellulose against the cathode in barbitalbuffer pH 8 6 ionic strength 0 1

88 faecal strains 96 wound strains and 23 autopsy strains were investigated 8 7 and 35 per cent respectively of the strains produced enterotoxin B 2 of the enterotoxin B negative strains in the autopsy material originated from patients with classical staphylococcal enterocolitis These two strains however were positive in cattest according to *Dolman & Wilson* (Canad Pbl Hlth J *31* 68 1940)

Lincoln K Department of Clinical Bacteriology University of Gothenburg Gothenburg

SOME RESULTS OF QUANTITATIVE URINE CULTURE IN 1961 1964

3271 ice transported clean voided urine samples from paediatric and medical patients with diagnosed or suspected urinary tract infections treated as well as untreated were investigated in 1961 196? 75 per cent of the samples came from females and 75 per cent came from patients younger than 16 years In infants aged one day to four weeks male samples were more numerous than female ones (155/93) which mirrors the higher susceptibility of male neonates to urinary tract infection

46 7 per cent of all samples contained $<$ 1000 28 1 per cent 1000–49 000 5 7 per cent 50 000–190 00 (= 10 ± 50 per cent) and 19 5 per cent 200 000 or more bacteria per ml of urine of the dominating bacterial species in the sample This was *E coli* in 67 9 per cent *Enterococci* in 8 3 per cent *Proteus* in 5 2 per cent *Klebsiella – Cloaca (Aerobacter) in 4 1 per cent Staph albus* in 3 3 per cent and *Pseudomonas* in 2 7 per cent of 637 samples with 200 000 or more bacteria per ml of urine

TRANSACTIONS OF THE SWEDISH PATHOLOGICAL SOCIETY

Meeting November 28, 1964

Received 12 i 65

Biberfeld P Ericsson J Perlman P & Raftell M SOME ULTRASTRUCTURAL AND IMMUNOLOGICAL FEATURES OF *IN VITRO* PROPAGATED RAT LIVER CELLS

In vitro propagated rat liver cells were studied using electron microscopic and immunological techniques in order to (1) try to confirm their origin from parenchymal cells and (2) investigate the ultrastructural and immunochemical alterations involved in the process of *in vitro* dedifferentiation and transformation

Hepatocytes from primary cultures of rat liver cells were cloned twice and propagated *in vitro* in spinner cultures as an established cell line (PAR cells) Using fluorescent antibody technique the PAR-cells were shown to react specifically with antiserum against isolated rat hepatic parenchymal cells they did not react with antiserum against other types of cells present in rat liver Lubrol DOC extracts of PAR-cells and freshly isolated hepatic parenchymal cells showed partially identical immunoelectrophoretic patterns with an antiserum against the parenchymal cells whereas no precipitin lines were obtained with antiserum against other types of rat liver cells

By electron microscopy the *in vitro* propagated cells showed local specialization of their plasma membrane with formations of *microvilli* These were most prominent on the free surfaces of cells growing in multicellular cords where structures resembling bile capillaries were observed

Such cells also displayed *stud like projections* of the plasma membrane and *intercellular junctions* (junctional complexes and desmosomes) *Glycogen* in particulate form (stainable with lead) was present in most of the cells The *Golgi apparatus* was often large and surrounded by numerous *multivesicular bodies cytosomes* and *cytosegresomes* Fine *cytoplasmic filaments* were present in many cells and were predominantly localized around the nucleus or immediately below the plasma membrane The nucleo-cytoplasmic ratio increased with the time of propagation due to decreased cytoplasmic volume One or several large *nucleoli* were often seen in the diffusely distributed nuclear chromatin

The immunochemical as well as many of the ultrastructural features of the *in vitro* propagated cells support the assumption that these originate from hepatic parenchymal cells The cells appeared to be moderately dedifferentiated and displayed several structural similarities with experimentally induced hepatomas

Ericsson J & Glinsmann W H ELECTRON MICROSCOPIC OBSERVATIONS ON FOCAL DEGENERATIVE CYTOPLASMIC ALTERATIONS IN LIVER CELLS INDUCED BY HYPOXIA

In preliminary experiments two changes were observed in the cytoplasm of rat hepatic parenchymal cells during hypoxia (*a*) universal swelling of the mitochon

Ampicillin particularly in large doses had a distinct therapeutic effect in experimental Haemophilus meningitis

The pathological changes in the meninges corresponded to the clinical picture In experiments with β streptococci and Haemophilus influenzae however mild changes were discernible even before signs of meningitis had appeared

Holm S F & Kaijser B *Department of Bacteriology University of Gothenburg* Gothenburg EXTRACELLULAR DPNASE ACTIVITY AND A CORRESPONDING INTRACELLULAR INHIBITOR IN BETA HAEMOLYTIC STREPTOCOCCI

The extra and intracellular DPNase activity of various strains of beta haemolytic streptococci (Lancefield s group A C and G) was studied Intracellular DPNase activity was not found in any of the strains tested although extracellular activity was detected in most strains The intracellular material from DPNase positive strains prepared by sonic treatment and freeze pressing (x pressing) inactivated the extracellular DPNase Intracellular material from DPNase negative strains gave no such inhibition The DPNase inhibition caused by intracellular material was not related to any particular type or group of streptococci since any of the DPNase positive strains could inhibit the enzyme activity

It was found that the inhibitor was heat sensitive (almost complete destruction after 10 minutes at 60° C) and trypsin labile The inhibitor was non dialysable and seemed to have a molecular weight of 20–40 000 according to gelfiltration experiments performed on Sephadex G 75

It was further found that the inhibitor acted as an antigen when tested in rabbits and that its corresponding immune serum blocked the inhibitory effect of the intracellular material

Bernander S Hallberg T & Edebo L Department of Bacteriology University of Uppsala Uppsala ON THE GROWTH OF CANDIDA ALBICANS IN DUBOS MEDIUM

When Candida albicans is inoculated in Dubos medium and incubated overnight at 37° C it grows in characteristic flocks while the surrounding medium is left clear Microscopically exuberant mycelial growth is seen to constitute the flocks while almost no yeast cells are observed in the surrounding Chlamydospore like cells are produced terminally on some hyphae

Since 1954 this property among Candida albicans strains from human sources has been investigated The identification of Candida albicans was done by observing chlamydospores on corn meal and/or taurocholate agar During one period fermentations and assimilations were performed with the same results Most strains of Candida albicans (80–90 per cent) grew in a characteristic flocky fashion Some strains (15 per cent) however formed very small flocks called grains This property was shared by half of the Candida tropicalis strains and a few other strains of closely related Candida species The remainder of these species yielded a smooth turbid growth

Our experiments indicate that Dubos medium or modifications of it is a valuable aid in the identification of Candida albicans They also demonstrate that both Candida albicans and Candida tropicalis are heterogenous species related to each other These facts have earlier been demonstrated serologically Preliminary experiments however indicate that growth characteristics and serological properties do not parallel each other

Stormby N & Wallenius K EXPERIMENTAL CANCER OF THE ORAL CAVITY IN HAMSTERS WITH REDUCED SALIVATION

Jonsson N & Sjogren H O FURTHER STUDIES ON THE ANTIGENICITY OF ROUS SARCOMA IN MICE

Sarcomas induced in mice by the Schmidt Ruppin variant of Rous sarcoma virus possess common tumour specific antigen(s) demonstrable by means of transplantation methods Animals pretreated with allografts of Rous sarcomas show a specific transplantation resistance against isografts of Rous sarcoma reflected in a reduced frequency of takes as well as in a slower tumour growth than in untreated controls. Pretreatment with allografts of non Rous tumours induced no specific resistance Sera from the animals pretreated with allografts of Rous sarcomas contained no virus neutralizing antibodies

In the course of the investigation it was found however that the antigenicity of the tumours diminished during isotransplantation Table 1 demonstrates the results of isografting of two Rous sarcomas (RSC early and late passage) and RSC57A (early passage) after pretreatment with allografts of tumour RYC (early and late passage) In this case and several other experiments pretreatment performed with allografts of late (15th 20th) passages thus gave a weak or no transplantation immunity compared with pretreatment with earlier passages Late tumour generations were also less susceptible to established transplantation immunity than earlier passages

Pretreatment with isografts of heavily irradiated tumour cells belonging to early but not late passages also gives a specific transplantation resistance. No individual tumour specific antigens could be demonstrated in addition to the group specific antigens

TABLE 1

Results of Isografting of Tumour RSC (Early and Late Passage) and RSC57A After Pretreatment with Tumour RYC (Early and Late Passage) Preirradiated Animals

Challenge tumour	Cell dose	Untreated	Allografted with RYC (early passage)	Allografted with RYC (late passage)
RSC 2 gen	5×10^3	5/5	2/5	5/5
RSC 21 gen	5×10^3	4/4	4 5	5/5
RSC57A 3 gen	5×10^2	5/5	1/4	3/5
	5×10^3	5/5	3/4	4/4

Ahlström C G & Mark J ROUS TUMOURS IN RABBITS

Rous sarcoma virus strain Schmidt Ruppin elicits fibrosarcomalike tumours in young rabbits (up to 1500 gms) after intramuscular or subcutaneous injection The tumours appeared 10–14 days after the inoc [illegible]

[illegible]

Many vessels in the lungs and l [illegible]
proliferation of cells similar t [illegible]
sh wed as the Rous sarcoma in chickens a high content of acid mucopolysaccharides [illegible] tumours

dria and (*l*) occurrence of cytolytic vacuoles (cytosegresomes) containing sequestrated cytoplasmic organelles apparently set aside for intracellular digestion The findings indicated that induction of hypoxic alterations might serve as a useful experimental model for the study of cytosegresome formation and also for investigations of the morphologic alterations in various organelles resulting from digestion with lysosomal enzymes (known to be present in cytosegresomes)

Hypoxia was induced by subjecting rats to a 5 per cent oxygen atmosphere for 2 hours Small liver pieces were fixed in OsO_4 and embedded in Epon for electron microscopy As a rule cytosegresome formation occurred in the close vicinity of the Golgi apparatus The membranes forming the walls of cytosegresomes were probably derived from closely associated endoplasmic reticulum or possibly from the Golgi system Early alterations in segregated organelles were densification of matrix most apparent in mitochondria Later the structure of enclosed organelles was obliterated by irregular densifications and focal ruptures of membranes The end product was dense and membranous materials similar to those present in cytosomes By studying a large number of different forms of cytosegresomes the sequential alterations during the degradation within cytosegresomes of various organelles could be followed The segregated organelles showed distinct patterns of alterations during degradation specific for each type of organelle thereby the enclosed structures could be classified with respect to origin even at relatively advanced stages of breakdown The findings strongly supported the theory that some *cytosomes originated from cytosegresomes as suggested previously concerning similar* structures in kidney tubule cells under normal conditions

Seljelid R & Ericsson J ELECTRON MICROSCOPIC OBSERVATIONS ON RENAL CARCINOMA IN MAN

In a correlated light and electron microscopic study of five renal adenocarcinomas tissue was obtained during surgery and immediately immersed in buffered formaldehyde *and osmium tetroxide In four cases the tumor tissue was predominantly* composed of *clear cells* as revealed by light microscopy The cytoplasm in these cells was strongly PAS positive Following diastase digestion of the sections only a narrow rim of PAS positive substance remained usually located along the free cell margins

By electron microscopy the tumor cells of clear cell variety were characterized by (*1*) large amounts of glycogen like granules (stainable with lead hydroxide) in the cytoplasmic ground substance (2) numerous clear vacuoles usually lacking luminal contents (3) numerous abnormal mitochondria and (4) local specializations of the plasma membrane *e g* brush border like elements (probably corresponding to the PAS positive diastase resistant material) and formation of pinocytosis vesicles with specific membrane coatings Similar appearances are noted in the cells of the proximal tubules The findings thus support the assumption that the tumor cells originate from proximal tubule cells

In one case the tumor was predominantly of *granular* cell type Preliminary observations suggested that these cells—in comparison with the clear cells—contained less glycogen and fewer vacuoles but greater numbers of mitochondria

Zettergren L RAT LIVER REGENERATION DURING TREATMENT WITH THE LIVER CARCINOGEN 3 METHYL-4 DIMETHYLAMINOAZOBENZOI

The results indicate that transformation of bovine cells by RSV does not involve visible chromosome alterations whereas transformation induced by SV40 is accompanied by profound damage and rearrangement of chromosomes

Vasell M HISTOPATHOLOGICAL AND SPUTUM CYTOLOGICAL STUDIES ON METAPLASIA AND DYSPLASIA IN THE BRONCHIAL MUCOSA OF PATIENTS WITH AND WITHOUT PULMONARY CARCINOMA

Surgical specimens from 50 cases of pulmonary squamous and undifferentiated carcinoma and 20 cases of primary pulmonary adenocarcinoma and autopsy specimens from a control series of 56 cases without primary lung cancer were studied by histopathology Sputum from 678 patients was studied cytologically with respect to metaplastic cells with and without nuclear atypia Of these cases 119 displayed pulmonary squamous or undifferentiated carcinoma and 20 primary pulmonary adenocarcinoma

The frequency of metaplasia was similar in the histological and cytological series for controls 34 per cent and 37 per cent respectively and for squamous undifferentiated carcinomas 88 per cent and 83 per cent respectively The frequency of metaplasia was the same in cancer cases with negative and positive cancer cytology (80 per cent and 83 per cent respectively)

The results of both the histopathological and the cytological investigation show the high frequency of bronchial epithelial metaplasia and dysplasia in primary lung cancer The observation of metaplastic and dysplastic cells in sputum may be a way to improve the detection of early lung cancer

Zajicek J & Tjernberg B TUMOUR CELLS IN LYMPH FROM LYMPH NODES CONTAINING TUMOUR METASTASES

Vasell M HISTOPATHOLOGICAL CLASSIFICATION OF DYSPLASIA AND CARCINOMA IN SITU CERVICIS UTERI
A COMPARISON BETWEEN SWEDISH AND AMERICAN PATHOLOGISTS

The increases use of gynecological cytology has actualized the question of which degree of epithelial atypia the diagnosis of carcinoma in situ is justified The existing confusing terminology makes an uniform treatment difficult

Most important is to obtain a god correlation between cytology and pathology as to 1) terminology and 2) the evaluation of the epithelial changes It would be desirable to treat dysplasias with an uniform method based upon the pathologist s grading of the histological picture

There is only one criteria agreed on by all pathologists the similarity of the microscopical cellular structure of carcinoma in situ and invasive carcinoma An increasing number of pathologists are now accepting also a differentiated form of carcinoma in situ

Nine cervical specimens have been reviewed at four Swedish and five American pathological departments The specimens were mostl [illegible]

[illegible] ... [illegible] ated and untreated As the diagnosis of sim

stainable with Colloidal Iron and Alcian Blue The tumours were larger and persisted for a longer period in cortisonized animals than in the controls A series of rabbits were injected with carcinogenic and non carcinogenic hydrocarbons in the thigh and Rous virus was given intravenously a few days later The virus localized to the site of the injected hydrocarbons and induced fibrosarcoma like tumours which however spontanoously regressed after some weeks The Rous tumours in rabbits could not be transplanted to new rabbits nor have we been able to recover the virus from tumours older than 3 weeks

Mark J CHROMOSOME ANALYSIS OF ROUS TUMOURS IN RABBITS

Detailed chromosome analysis was carried out on 13 Rous tumours induced by intramuscular injection of Rous sarcoma virus strain Schmidt Ruppin into 1-3 week old albino rabbits The analysis was done on small fragments from the tumours cultivated *in vitro* for 4 6 days 50 or more metaphases and about 25 anaphases were analysed from each tumour The results from 4 rabbit tumours are given in the table Positive proof of chromosomal deviations from the normal rabbit karyotype was not found in any of the tumours The few aneuploid cells are regarded as squash artefacts and the few structural abberations are probably an *in vitro* effect

Litter	Rabbit	*Number of cromosomes*							Number of breakages
		43	44	45	86	88	176	N	
	RKM 78	3	58			5		66	3
I	RKM 79		47	1		3		51	1
	RKM 81		53		1	1	1	56	1
II	RKM 83	1	46	1		2		50	3
	Total	4	204	2	1	11	1	223	

Lithner F CHROMOSOME ANALYSIS OF BOVINE FIBROBLASTS EXPOSED TO SIMIAN VIRUS 40 (SV_{40}) AND ROUS SARCOMA VIRUS (RSV) *IN VITRO*

Bovine fibroblasts exposed to SV_{40} or RSV undergo morphologic transformations characteristic for each virus Metaphases from such cultures and unexposed control cells have been analyzed concentrating on long term results

Control cultures showed 52 per cent abnormal metaphases in passage 36 and 100 per cent in passage 63 The abnormality consisted of an increased number of metacentric and submetacentric chromosomes and a corresponding decrease in the number of acrocentrics as if the latter had fused to form the extra submetacentric and metacentric chromosomes

Cultures transformed by RSV showed no abnormalities not found in the ocntrols Samples from a comparatively early passage showed fewer abnormal metaphases (13 per cent) in the RSV than in the control cultures (52 per cent)

Cultures transformed by SV_{40} showed pronounced chromosome changes not seen in control cells *Already shortly* after exposure to virus a disproportionate increase of submetacentric and metacentric chromosomes was found with many total counts in the hyperdiploid range In addition pathologic forms were seen *i e* dicentric chromosomes and chromatid breaks

these cases using microradiography and serial sections These anastomoses are most often H shaped and have an inner diameter of 50 to 150 microns and a tortuous course They are found at the level of the lobular bronchi and resemble closely the "isolated" anastomoses found in congenital heart disease This resemblance and the fact that anastomoses were found in a case where the thrombi were nor mote than two weeks old suggest that the anastomoses may result from an opening up of pre existing non functioning precapillary connections

In 27 cases with normal lungs filling of peripheral pulmonary arteries was obtained in only 2 cases using the same technique

Bäckström C G Linell F & Östberg G CYSTIC MYXOMATOUS ADVENTITIAL DEGENERATION OF THE RADIAL ARTERY WITH DEVELOPMENT OF GANGLION IN THE CONNECTIVE TISSUE

Henrikson H Hagqvist G R Stenström B & Wallerström A TWO CASES OF RHINOSCLEROMA

Berge Th SPLENOMA

Saldeen T & Voigt G LACERATION OF SUPERFICIAL CEREBRAL VEINS

ilar changes may vary also among pathologists using the same terminology the severe dysplasias should be treated in the same way as carcinoma in situ in order to obtain an uniform therapy

Bergstrand A Hellman B & Nathorst Windahl G ELECTRON MICROSCOPIC STUDIES ON GLOMERULAR CHANGES IN MICE WITH OBESITY HYPERGLYCAEMIC SYNDROMA

Jonsson L THE PANCREATIC TISSUE AFTER LIGATION OF THE PANCREATIC DUCT IN RATS

In order to investigate the pancreatic tissue reactions and to make a comparative study atrophic pancreas containing islets tissue from rats with pancreatic duct ligation was implanted in the anterior chamber of the eye Two types of duct liga tion were used In the one— subtotal —the pancreatic ducts opening into the choledochus and duodenum were ligated near their outlets In the other— partial —the ligature was applied to the lienal part of the pancreas approximately between its middle and lienal third

Most of the rats were sacrificed five weeks later some six or twelve months after the operation Histological and histochemical examinations were made

The acinar tissue was replaced by adipose and connective tissue with dilated ducts and the islets of Langerhans Large islets were fairly infrequent but there seemed to be a rather high number of small irregular ones The number of islets seemed to be increased but the atrophic tissue is more compact and therefore the islets are lying closer

The α cell incidence in the islets of the atrophic tissue was low 15 6 per cent after partial and 15 1 per cent after subtotal ligature In normal pancreas the α cell incidence was 27 7 per cent The difference is significant

No explanation can be give to the low α cell incidence in atrophic tissue The α cells are well represented in grafts 6 months or 1 year old

Angervall L & Save Soderbergh J MICRO ANGIOPATHI IN THE GASTRO INTESTINAL TRACT IN JUVENILE DIABETICS

Bostrom K & Hassler O MICRO RADIOGRAPHIC STUDIES ON ARTERIAL CALCIFICATIONS

Hassler O INTRACRANIAL CALCIFICATIONS IN MAN

Orell S & Hultgren S ARTERIAL BRONCHOPULMONARY ANASTOMOSES IN CHRONIC THROMBOEMBOLIC OCCLUSION OF PULMONARY ARTERIES

16 autopsy cases with thrombi—probably of embolic origin—in different stages of organization occluding major pulmonary arteries were investigated by injecting a thick suspension of Micropaque" into the bronchial arteries The injection time was kept short to avoid filling or capillaries and veins In 13 of the cases filling of pul monary arteries peripherally to thrombot c occlusions was obtained Precapillary anastomoses between bronchial and pulmonary arteries have been verified in 6 of

coloured deep reddish in polarized light The same granules react positive with 5 nucleotidase and acid phosphatase These histochemical reactions correspond to those of the infantil AFI

By electromicroscopic examination the storage substances are of irregular and different sizes The largest bodies we have named myelin like bodies because of their structures which are identic with those of the myelin sheath Beside these smaller osmophilic bodies with lamillar structure of variable density occur (Dtsch Z Nervenheilk In press)

Meeting November 12, 1964

Eng J & Lislrup J C THE DIAGNOSIS OF *MYCOPLASMA PNEUMONIAE* INFECTIONS BY MEANS OF COMPLEMENT FIXATION TESTS

A general survey was first given of *Mycoplasma pneumoniae* (Eaton agent) and its importance in lower respiratory tract infections in man Some observations were then discussed regarding the growth of Mycoplasma pneumoniae in liquid medium and the preparation of complement fixing antigen It was found that extraction of Mycoplasma pneumoniae suspensions with *ether* reduced the anticomplementary activity of some of the batches Further studies on the ether soluble Mycoplasma pneumoniae antigen are now in progress Finally the CF results in a selected series of 23 patients from the Oslo area hospitalized with clinically atypical pneumonia were reported In three of the 23 patients a significant rise in the CF titer was demonstrated and in one patient a very high titer (2560) was found in a single blood sample

Refsum S B d y EXFOLIATIVE CYTOLOGY

Listrup J C RESPIRATORY SYNCYTIAL VIRUS A REPORT OF TWO EPIDEMICS FROM THE OSLO AREA

Published in Nordisk Medicin 72 1324–1326 1964

Meeting December 17, 1964

Lie S MICROBIAL GENETICS AND RECOMBINATION OF GENETIC PROPERTIES IN *NEISSERIA MENINGITIDIS*

A brief outline of the progress in microbial genetics the last years was given in order to show how studies of micro-organisms have led to the concept of molecular genetics and hence to the problem of the genetic code The rII system of bacteriophage T4 with its principals and methods of genetic analyses and mapping were chosen as an example A recent map showing the distribution of more than 300 mutants in this region of *ca.* 2 000 base pairs was discussed The problem of the genetic code was illustrated by the experiment of *Crick and coworkers* (1961) in which the behaviour of acriflavin induced rII mutants was studied This work gave the first strong support to the *idea that the* coding unit is the sequence of three bases the socalled triplet The last two years research on *in vitro* protein synthesis with synthetic messenger has given us the possible triplets for each of the 20 amino

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TRANSACTIONS OF THE PATHOLOGICAL SOCIETY OF NORWAY

Oslo, Norway

Meeting October 8, 1964

Received 14 ii 65

Godal T IMMUNOLOGICAL ASPECTS OF VIRAL CARCIOGENESIS

Immunological fenomena in the carciogenesis of Polyoma virus (PV) in syrian hamsters was reported with occasional attention to other oncogenic viruses Tumors induced by PV or other oncogenic viruses may be free of infectious virus This observation provoke two alternatives in viral carcinogenesis

1 Does the virus initiate a process which continue by itself without integration of genetic material from the virus in the tumor cell (the kick and run hypothesis) or
2 is a partial or total integration of the viral genome required in order for the mammalian cells to persist as tumor cells?

From this point of view some recent findings was analyzed as summarized subsequently

In PV induced virusfree tumors of hamsters have been demonstrated antigens with viral rather than host cell specificity This suggests that the antigen is coded by a viral gene and indicates that genetic material of the virus persist in tumor cells

Virus induced tumors may be carried and transferred in adult animals for years without loss of specific antigens In immunological competent animals should malignant cells without specific antigens have a selective advantage Since such a selection appearantly not occure the observation indicates that the surviving genetic material of the virus is intimately associated with the malignant properties of the neoplastic cell On this basis attention was payed to alternative 2 as the most plausible of the alternatives cited above

Koppang N JUVENILE AMAUROTIC IDIOCY BY ENGLISH SETTER IN NORWAY

The dogs develope normally the first year Than gradually a visual failing and an alteration in their behavior and general condition occur They emaciate and dehydrate About the age of 2 years they die often in an attack of general convulsions The illness is hereditary

In a comparative study of 19 dogs with sphingolipidosis and brain tissue from 4 human cases one with infantil and 3 with juvenile amaurotic family idiocy (AFI) it has been shown that the morphological changes and the histochemical reactions tests by these dogs and the human juvenile AFI are almost the same (Proc 9 Nord Veterinærmøde København II 862 867 1962 Ergebn allg Path Anat 47 In press) The small and big pyramidic cells of hippocampus the motor cells of the lumbar part of spinal cord contain some very small granules These are soluble in alcohol partly double refractile, and in frozen section stained with Sudan black they are

Acta path et microbiol scandinav 64 161–166 1965

The Medical Department B (Head Professor O Storstein M D)
and the Institute of Pathological Anatomy (Head Professor O Torgersen M D),
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SUCCINIC DEHYDROGENASE ACTIVITY AND HISTOLOGICAL CHANGES OF THE SMALL INTESTINAL MUCOSA IN PATIENTS WITH GASTROINTESTINAL DISEASES

By

J MYREN, E GJONE and S SKREDE

Received 3 ix 64

Histological changes of the small intestinal mucosa have been extensively studied (*Salvesen & Skogrand* 1957, *Himes & Adlersberg* 1958, *Shiner* 1959, *Shiner & Doniach* 1960, *Thurlbeck et al* 1960, *Girdwood et al* 1961, *Holmes et al* 1961, *Shiner & Birbeck* 1961) Usually the changes are severe in cases with idiopathic steatorrhoea, whereas they are slight in patients with other diseases affecting the gastrointestinal tract Thus *Shiner* (1959) found total or subtotal atrophy of the mucosal villi in 21 out of 23 cases with idiopathic steatorrhoea, whereas only two out of 52 patients with partial gastrectomy, pancreatitis and other diseases had severe changes of mucosal villi Similar results have been reported by *Shiner & Doniach* (1960), *Thurlbeck et al* (1960) and *Girdwood et al* (1961)

The finer structure of the intestinal epithelium has been examined by electron microscopy by *Zetterquist & Hendrix* (1960), *Ashworth et al* (1961) and *Sherman et al* (1961) Severe changes were revealed in the epithelium from cases with idiopathic steatorrhoea, whereas the changes in other diseases were slight

Functional changes in the small intestinal mucosa as evidenced by enzymatic activities seem to have been little examined A morphological and histochemical analysis of the human epithelium is reported, however by *Padykula et al* (1961) These authors also examined succinic dehydrogenase activity (SDH) by means of Neotetrazolium and Nitro BT and found that the SDH activity was decreased in patients with idiopathic steatorrhoea

Spiro et al (1964) examined a series of enzymatic activities including SDH glucose-6 phosphate (G-6 PDH) deh- ...
TPN ...

acids Various experiments were reported which support the assumption that the code is universal, *i e* the same for all biologic species One of the most convincing arguments for this hypothesis is the work of *Trautner & Abel* (1964) who succeeded in growing vaccinia virus on the bacterium *Bacillus subtilis*

In the last few years, the speaker has been engaged in studies on the genetics of *Neisseria meningitidis* The problems encountered with in establishing a genetic system in this species were discussed and some of the results presented Growth of two geno typically distinct meningococcal types in mixed culture results in the pro duction of numerous recombinants, and it was shown how this could be explained by various phenomena concerning the transformation process in this species The role played by these genetic mechanisms in the evolution and adaptability of this species was finally briefly discussed

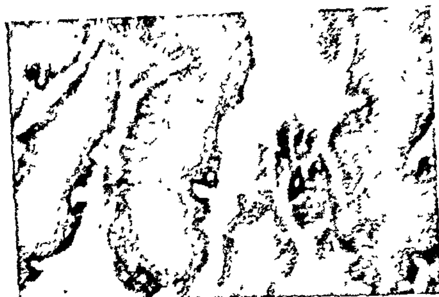


Fig 2

Section showing SDH activity ++ (decreased) Man 54 years old with partial gastrectomy performed 10 years previously Black granules unevenly distributed in the epithelial lining of the villi (normal histological findings) MTT technique × 200

Category I Flat mucosal surface with no normal villi Increased cellular infiltration of lamina propria

Category II ...

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RESULTS

A *severe decrease* in SDH activity (degree of SDH activity +, Figs 3 4 5) was found in 6 of the 17 patients Two of these had idiopathic steatorrhoea In *one* a partial gastrectomy had been performed and three patients had other diseases

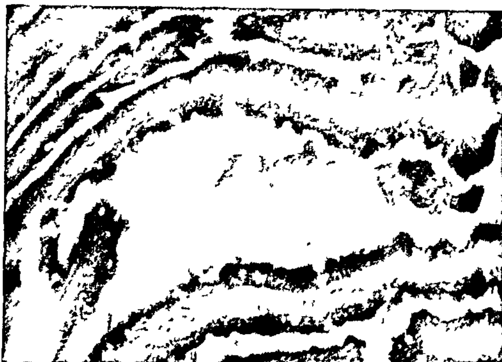


Fig 1

Cryostat section of biopsy from small intestinal mucosa SDH activity +++ (normal) Man 30 years old with chronic pancreatitis Black granules evenly distributed most marked at the surface border of the epithelial cells of the normal villi MTT technique × 200

activity and suggested that the decrease in enzymatic activity was secondary to the changes observed in histological sections

Some observations in animals, however, seem to indicate that inhibition of dehydrogenase activity may be found without obvious morphological alterations being visible by light microscopy (*Lukelic et al* 1964, *Myren et al* 1964) The present report may show that a discrepancy between enzymatic activity as evidenced by SDH, and morphological findings may also be found in humans with gastrointestinal diseases

MATERIAL AND METHODS

Biopsy specimens were obtained from the small intestine of 17 patients by a *Crosby* capsule The average age of these cases was 48 years (30 66 years) Four of these cases had idiopathic steatorrhoea in four a partial gastrectomy had been performed 10 years previously and four had chronic pancreatitis Of the remaining 4 patients one had Crohn s disease one had Whipple s disease one was observed for scleroderma and one suffered from secondary amyloidosis The diagnosis was based on clinical findings and on glucose and d xylose tolerance tests vitamin A absorption test the Schilling test X ray examinations and on determinations of fat in the stools

Histology The biopsies were divided into two parts of which one was fixed in 4 per cent formalin embedded in paraffin and stained with haematoxylin and eosin The changes were evaluated by microscopy according to three categories (*Girdwood et al* 1961)

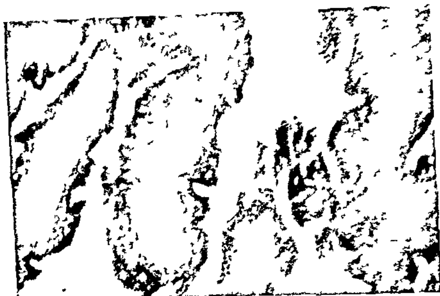


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Category 1 Flat mucosal surface with no normal villi Increased cellular infiltra

only few goblet cells

SDH act vity The other half of the specimens was frozen on dry ice of CO_2 immed ately after removal from the biopsy capsule Cryostat sections six to seven microns thick were cut and incubated in a med um containing MTT (3 (4 5 d methyl thiazolyl 2) 2 5 diphenyl tetrazolium bromide) and sod um succinate as described by *Pearse* (1960) By th s method f ne black granules are precipitated in the cell

+ decreased) when the amount of black granules was less than in the previ us category (Fig 2) The patchy or evenly reduced staining was most pronounced at the luminal surface of the epithelial cells of the villi The degree of activity was designated + (severely decreased) when the black colouring was much weaker than that des gnated +++ in all parts of the epithelial l ning

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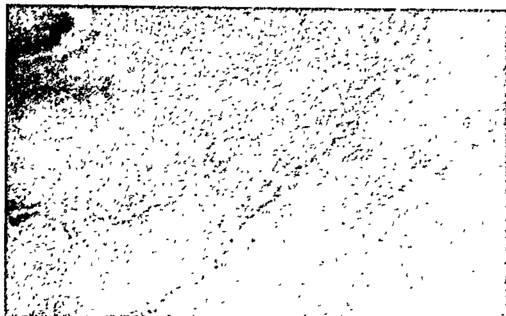


Fig. 3.
Section from intestinal mucosa. SDH-activity + (much reduced) in the epithelium of short and swollen villi. Man, 45 years old, with Whipple's disease. MTT-technique × 200.

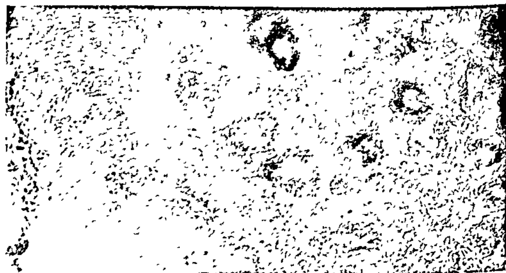


Fig 4.
Section from biopsy of small intestinal mucosa. SDH-activity + (much decreased) in the epithelium of flat mucosal surface without villi. Man, 50 years old, with idiopathic steatorrhoea. MTT-technique × 200

Severe histological changes were found in all of these patients The biopsy specimens showed short villi in four and flat villi in two cases. A heavy cellular infiltration was found in the lamina propria in all instances.

A decreased SDH-activity (degree of activity + +, Figs. 2, 5) was

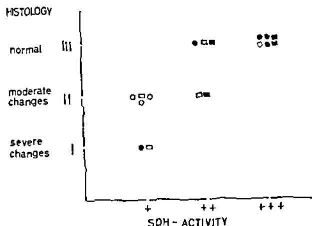


Fig 5

Correlation between the degree of histological changes (III I) and the degrees of SDH activity (+++ to +) in biopsy specimens from 17 patients Out of these cases four had idiopathic steatorrhoea (□) four had chronic pancreatitis (■), five had partial gastrectomy (●), and four had other diseases (○)

found in 5 patients Two of these cases had idiopathic steatorrhoea, two had chronic pancreatitis and one had a partial gastrectomy.

The histological picture was normal, however, in three of these cases One of these had been treated for idiopathic steatorrhoea with a gluten free diet (fecal fat 10 g and nitrogen 2 g per 24 h). In the two remainin patients abnormal villi were found

A *normal* SDH-activity (degree of SDH-activity + + +, Figs 1 and 5) was found in 6 patients Two of these had chronic pancreatitis, three a partial gastrectomy and one amyloidosis In all these cases the histological picture in haematoxylin and eosin stained sections was completely normal

DISCUSSION AND CONCLUSIONS

Although this small series of cases does not allow any definite conclusions, the results seem to indicate that a fairly good relationship exists between the SDH activity and the degree of histological changes in the mucosa of the small intestine as also stressed by others (*Spiro et al* 1964)

A decrease in SDH-activity was found, however, *in the villous epithelium* of some of the cases in which the histological picture was completely normal, in one instance even in idiopathic steatorrhoea in remission In the stools of this patients the content of fat and nitrogen was slightly higher than normal This finding may indicate that morphological findings and enzymatic activity may show discrepancies A larger series of cases, however, is necessary to show the importance of dehydrogenase activity for absorption

SUMMARY

A comparison between the degree of histological changes and succinic dehydrogenase activity (SDH activity) in biopsies of small intestinal mucosa has been performed in 17 patients Four of these cases had idiopathic steatorrhoea, five had partial gastrectomy four chronic pancreatitis, and four patients had other diseases

The SDH activity was examined in cryostat sections by the method of *Pearse* (1960) with MTT

A fairly good correlation was found between the SDH activity and the histological changes (Fig 5) A decrease in SDH activity was found however, in some cases which showed a completely normal histological picture

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Acta path et microbiol scandinav 64, 167–175 1965

The Influenza Department, Statens Seruminstitut Copenhagen

THE INFLUENCE OF STEROID HORMONES AND GROWTH HORMONES ON THE EFFECT OF INTERFERON IN TISSUE CULTURE

By

V Reinicke

Received 20 xi 64

Experiments by *Kilbourne et al* (8) and previous studies in this laboratory (16) have shown that the virus inhibiting effect of preformed interferon assayed in chorioallantoic membranes infected with influenza virus decreased when the chorioallantoic membranes had been pretreated with steroid hormones Results obtained in tissue culture experiments by *De Maeyer & De Maeyer* (15) employing a continuous line of rat tumour cells infected with Sindbis virus did not confirm the findings obtained with chorioallantoic membranes They found that addition of cortison and metandienonum to tissue cultures used for interferon assay was without influence on the virus inhibiting effect of interferon or in certain instances even enhanced the effect of interferon It seemed of interest therefore to investigate the influence of steroid hormones on the effect of interferon in tissue cultures derived from the same source as chorioallantoic membranes, *i e* the chick embryo Steroids known to possess varying biological properties *in vivo*, *i e* catabolic, anabolic, adrogenic or oestrogenic effects were assayed in an attempt to dem-

... to increase protein synthesis *in vivo* (3, 10) were included in the study

MATERIALS AND METHODS

Virus ...
was em...

... fibroblast monolayers grown ... with approximately 10^3 plaque forming units (p f u) of Sindbis virus After 36 to 48 hours of incubation at 36° C when the cytopathogenic effect was

The author wishes to thank Mrs *B Saugbjerg* for skilled technical assistance

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a[illegible] chorioallantoic membranes, i e the chick embryo Steroids known to possess varying biological properties *in vivo*, i e catabolic, anabolic, adrogenic or oestrogenic effects were assayed in an attempt to demonstrate a possible correlation between the effect of the hormones *in vivo* and *in vitro* Two different growth hormones which like certain steroid hormones are known to increase protein synthesis *in vivo* (3, 10) were included in the study

MATERIALS AND METHODS

Virus [illegible]
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EID50 ml[illegible]
Sindbis [illegible]
New Yor[illegible]
laborator[illegible]
human a[illegible]
Sindbis [illegible]
grown in [illegible] [illegible] monolayers [illegible] with approximately 10^3 plaque forming units (p f u) of Sindbis virus After 36 to 48 hours of incubation at 36° C when the cytopathogenic effect was

The author wishes to thank Mrs *B Saugbjerg* for skilled technical assistance

almost complete the cell sheets were scraped down with a rubber policeman The cellular debris was disintegrated and mixed with the medium by vigorous pipetting and the mixture was shell frozen in 1 1 ml amounts in a dry ice alcohol bath and stored at —60° C Sindbis stocks prepared in this manner contained 2×10^8 to 8×10^8 p f u per ml when titrated in chick embryo fibroblast cultures

Media

Growth medium for tissue cultures consisted of medium 199 containing 5 per cent calf serum, 4 per cent chick embryo extract and sodium bicarbonate Medium for cultures grown in bottles contained 0 08 per cent bicarbonate, medium for cultures grown in Petri dishes contained 0 19 per cent bicarbonate

Maintenance medium was identical with the growth medium except that no chick embryo extract was included

Earle's BSS was employed for washing of monolayers, washing of pieces of chick embryo tissues during preparation of cultures and as diluent for interferon

Hanks BSS was used for preparation of *chick embryo extract* in the following manner 10 to 11 day-old white Leghorn chick embryos were decapitated and washed in Hanks BSS The embryos were weighed and Hanks' BSS was added until 2 5 times the original weight was reached The mixture was homogenized in a Waring blender at alternating low and high speed cycles of two to three minutes duration The mixture was left for half an hour and subsequently centrifuged for 10 minutes at 2500 r p m The supernatant was stored at —20° C and recentrifuged immediately before use

Phosphate buffer at pH 7 38 containing 0 2 per cent bovine albumin (4) was used as diluent for Sindbis virus

Citrate hydrochloride buffer at pH 4 was used as solvent for growth hormones and *glycerine hydrochloride buffer* at pH 2 was employed for inactivation of influenza virus

Trypsin A 0 2 per cent solution of trypsin (Nutritional Biochemicals Corporation Cleveland Ohio USA) in phosphate buffer was employed

Overlay medium consisted of medium 199 (without phenol red) containing 5 per cent calf serum 5 per cent chick embryo extract 0 19 per cent sodium bicarbonate and 0 005 per cent neutral red *For preparation of overlay the medium was kept at* 37° C and immediately before use mixed with equal parts of 1 8 per cent Noble agar in Earle's BSS (without phenol red) At the time of mixing of medium the agar solution was kept at a temperature of 40° C after being liquified by boiling

Antibiotics G penicillin and streptomycin were added to all media and diluents giving a final concentration of 200 units/ml and 50 γ/ml respectively

Hormones

Hydrocortisone A highly soluble compound di (17 hydroxycorticosterone 21) phosphoriacidester sodiumsalt (16) was employed dissolved in maintenance medium

d Aldosterone 17α methyl 17β-hydroxy andosta 1 4 dien 3 on (metandienonum (NFN) ∾ Dianabol) *testosteroneisobutyrate* and *oestradiol 17 ben oate* were ob

before use the compounds were

iol and mixed with maintenance

usually did not exceed 0 02 per

cent Appropriate control medium containing the same amount of ethanol were included in the experiments

f ma n

Growth hormones (GH) obtained from pituitary glands of sheep [illegible] d

[illegible]

fer at pH 4

The hormones were kindly supplied by the following Hydrocortisone Dr *H Vetmehren* Frederiksberg Chemical Laboratories Ltd Copenhagen Denmark d Aldosterone and metandienonum Dr *Jørgen Lorenzen* CIBA Societe Anonyme Basel Switzerland Testosteroneisobutyrate and oestradiol 17 benzoate Leo Pharmaceuti

cal Products Copenhagen Denmark, Growth hormones Dr *F Paulsen*, Fetring AB, Malmø, Sweden

Tissue Culture

Tissue cultures were grown either in Pyrex glass T bottles with flat sides each having an area of approximately 8 cm × 5 cm or in plastic Petri dishes measuring 8.5 cm in diameter The plastic Petri dishes were obtained from A/S Nunc Trading Roskilde Denmark.

consecutive five minutes cycles After each cycle the supernatant fluid containing the liberated cells was removed a small amount of calf serum was added and the mixture cooled in an ice bath The cells were spun down at 700 r p m for 10 minutes and resuspended in growth medium Following staining with 0 1 per cent crystal

Interferon

... the virus (2) followed by 24 hours dialysis against Earle s BSS to restore a pH of approximately 7 Inactivation of virus was ascertained by haemagglutinin and infectivity titrations (16) Allantoic fluid pools treated in this manner were used as batches of interferon

Titration of interferon ... inhibition technique rathe ...

... as the curve ... $\frac{n_2}{n_1} = 1$ should be used when two plaque titrations employing equal numbers ... cultures are being compared the curve marked $\frac{n_2}{n_1} = 2$ should be used when com

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d Aldosterone *17α methyl 17β hydroxy andosta 1 4-dien 3 on* (*metandienonum* (NFN) ∾ Dianabol) *testosteroneisobutyrate* and *oestradiol 17 benzoate* were obtained as pure crystalline preparations Immediately before use the compounds were dissolved in a minute amount of 99 9 per cent ethanol and mixed with maintenance medium The concentration of ethanol in the media usually did not exceed 0 02 per cent Appropriate control medium containing the same amount of ethanol were included in the experiments

The above mentioned hormones were chosen as typical representatives of main groups of steroid hormones *i e* catabolic steroids steroids with main effect on electrolyte balance anabolic steroids androgenic steroids and oestrogenic steroids

Growth hormones (GH) obtained from pituitary glands of sheep (Prolactin) and swine (Somacton) with an activity of 20 IU/mg and 1 IU mg respectively, were employed Immediate before use the hormones were dissolved in small amounts of citrate hydrochloride buffer at pH 4 and mixed with maintenance medium Control medium consisted of maintenance medium containing an appropriate amount of buffer at pH 4

The hormones were kindly supplied by the following Hydrocortisone Dr *M Vermehren* Frederiksberg Chemical Laboratories Ltd Copenhagen Denmark d Aldosterone and metandienonum Dr *Jorgen Lorenzen* CIBA Société Anonyme Basel Switzerland Testosteroneisobutyrate and oestradiol 17 benzoate Leo Pharmaceuti

cided to use the same period of preincubation also in the tissue culture experiments in order to obtain maximal effect on the cells by prolonged exposure to the hormones.

In the first series of experiments the effect of 24 hours of incubation with hormones on the tissue culture cells as regards susceptibility to Sindbis virus was studied. The experiments were performed with the maximal concentrations of the hormones employed in this study.

TABLE 1

The Influence of 24 Hours Preincubation with Hormones on Sindbis Virus Plaque Titres in Chick Embryo Fibroblast Cultures

Type of hormone	Concentration of hormone (γ/ml)	Number of plaques (average of six cultures)
Hydrocortisone	250	63.7
Control		69.5
Metandienonum	50	28.8
d Aldosterone	50	31.7
Control		30.7
Growth Hormone (20 IU/mg)	50	36.2
Growth Hormone (1 IU/mg)	50	35.8
Control		33.7
Testosterone	50	62.2
Oestradiol	50	75.4
Control		72.0

TABLE 2

[illegible]

Preincubation with	Number of plaques (average of 5 cultures) obtained in dilutions of interferon					Titre of interferon
	1:4	1:8	1:16	1:32	Earle's BSS	
Hydrocortisone 50 γ/ml	0	7.6	21.8	27.8	26.8	1/11
Hydrocortisone 50 γ/ml followed by washing	0	8.5	23.0	31.8	31.2	1/12
Hydrocortisone 10 γ/ml	0	9.5	22.2	28.6	29.0	1/11
Control	0	7.5	19.3	29.0	26.0	1/12

Confluent monolayers of chick embryo fibroblast cells grown in Petri dishes had their growth medium replaced by hormone containing maintenance medium. Six cultures were used for each type of hormone. After 24 hours incubation at 36° C the cultures [illegible] the [illegible] tion [illegible] the [illegible] cel [illegible] of Earle's BSS and subsequently inoculated

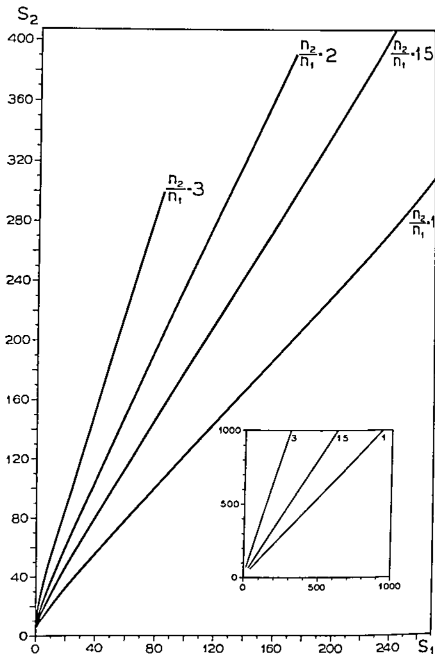


Fig 1

Diagram showing limits of significance of a Sindbis virus plaque titration system
Signatures See text

parisons are made between two titrations the one of which is employing twice as many cultures as the other one and so on A following paper describes details concerning the statistics of the titration system (12)

EXPERIMENTAL RESULTS

In earlier studies (16) on the influence of hydrocortisone on the titres of interferon assayed in chorioallantoic membranes 24 hours preincubation of the membranes with hydrocortisone was employed It was de

the simultaneous presence of hormone and interferon during the titration would influence the degree of inhibition compared to that obtained when only interferon was present Tables 2, 3, 4 and 5 summarize the results of these experiments It can be seen that in the present assay the interferon titres were identical whether or not the cultures had been preincubated with hormones The experiments with washed cultures show that identical titres were also obtained whether the cultures contained interferon plus hormone or interferon only during the assay The experiments recorded in Tables 3 and 4 employing preincubation with 250 γ hydrocortisone and 50 γ testosterone, respectively, show slightly depressed plaque titres possibly due to a slight cell toxic effect of the hormones The titres of interferon, however, were also in these experiments identical with titres obtained in the control cultures

TABLE 5

The Influence of 24 Hours Preincubation with Metandienonum d Aldosterone and Growth Hormones on the Plaque Inhibiting Effect of Interferon in Chick Embryo Fibroblast Cultures No Washing of Cultures was Performed after the Preincubation

Preincubation with	Number of plaques (average of 6 cultures) obtained in dilutions of interferon	
	1 4	1 8
Metandienonum 50 γ/ml	4 2	9 0
d Aldosterone 50 γ ml	4 0	10 7
Control	3 3	10 2
Growth Hormone (20 IU/mg) 50 γ ml	4 5	10 8
Growth Hormone (1 IU /mg) 50 γ ml	5 0	10 0
Control	3 2	10 7

DISCUSSION

The present experiments have shown that preincubation with steroid hormones and two growth hormones did not influence the virus inhibiting effect of interferon when assayed in chick embryo fibroblast cultures infected with Sindbis virus These findings obtained in tissue cultures disagree with observations by *Kilbourne et al* (8) and with previous findings in this laboratory (16) on the effect of interferon on influenza virus infected chorioallantoic membranes pretreated with steroid hormones These studies both showed that the effect of interferon was suppressed by hormones in this test system, *i e* the titre of a standard solution of interferon was lower when the assay was carried out in steroid pretreated chorioallantoic membranes

The present observations are also in a certain disagreement with the observations by *De Maeyer & De Maeyer* (15) on the influence of cortisone and metandienonum on the effect of interferon in tissue culture These authors employed a continuous line of rat tumour cells and the

with approximately 60 p f u of Sindbis virus, the amount varying fi
30 to 70 p f u through the experimental series After virus adsorp
for 1 hour the cultures were overlayed and plaques were read afte
hours incubation The results appear from Table 1, which shows
the hormones employed did not significantly influence the plaque ti
obtained in the tissue cultures (12) The titre obtained in the te
sterone preincubated cultures was slightly lower than the titre obtai
after preincubation with oestradiol but the difference from the titr
the control was only on the borderline of significance (12) It
furthermore observed that at the time of plaque-reading all the ti
cultures seemed in good condition having taken up dye equally v

TABLE 3

The Influence of 24 Hours Preincubation with Varying Doses of Hydrocortison Plaque Inhibition Titres of Interferon in Chicl Embryo Fibroblast Cultures

Preincubation with hydrocortisone	Number of plaques (average of 5 cultures) obtained in dilutions of interferon				Titr interf
	1:8	1:16	1:32	Earle's BSS	
250 γ/ml	5 0	14 0	17 5	16 1	1/1
100 γ/ml	6 3	23 2	26 2	25 6	1/1
50 γ/ml	9 0	22 8	27 2	29 2	1/1
10 γ/ml	9 5	20 1	26 2	26 4	1/1
Control	9 8	22 8	31 0	26 8	1/1

TABLE 4

The Influence of 24 Hours Preincubation with Testosterone and Oestradiol on Pl Inhibition Titres of Interferon in Chick Embryo Fibroblast Cultures

Preincubation with	Number of plaques (average of 6 cultures) obtained in dilutions of interferon				Titr interf
	1:32	1:64	1:128	Earle's BSS	
Testosterone 50 γ/ml	32 2	51 3	64 3	62 1	1/3
Oestradiol 50 γ/ml	50 3	77 6	110 8	109 3	1/3
Control	44 5	74 2	111 0	105 6	1/4

Obviously, the employed hormones did not have any severe to
effects (1, 9) on the cells of the monolayers This observation is in
cordance with earlier experiments (19) showing that infectivity tit
of influenza A virus grown in hormone pretreated chick embryo fib
blast cultures were not different from those obtained in control cultu

In the subsequent series of experiments batches of interferon w
titrated in monolayers pretreated for 24 hours with varying concent
tions of hormones Two fold dilutions of interferon were titrated in 5
6 cultures per dilution after removal of the hormone containing medi
and one washing with 10 ml of Earle's BSS per culture In some expe
ments washing of the cultures was omitted in order to examine wheth

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De Maeyer & De Maeyer (15) suggest that their findings of higher titres of interferon in tissue culture treated with steroid hormone possibly could be due to steroid induced change in the permeability of the cell membrane allowing the interferon to pass more easily into the cell interior In the present experiments obviously no such permeability changing effect can be considered The fact, however, that the effect of steroid hormones at the cellular level as well as the mode of action of interferon are still incompletely understood though extensive efforts have been directed towards both problems (5, 6, 7, 11, 13, 14, 18, 20 and others) makes the discussion of the influence of hormones on the effect of interferon rather hypothetical

It should, however, be pointed out that the experimental conditions employed for the studies on the effect of steroids on interferon titres differ considerably The chorioallantoic membrane assay thus uses reduction of yield of myxo (influenza) virus haemagglutinin for measurement of interferon while the plaque inhibition assay uses reduction of yield of infectious arbor (Sindbis) virus Furthermore, the chorioallantoic membrane technique employs allantoic cells maintained in situ whereas the plaque method depends on chick embryo cells maintained in tissue culture It seems not unlikely that these differences in experimental condition may account for the diverging results

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in a given species This point was theoretically mentioned by *Mitchell* (1948), by *Bullough* (1950), and later by *Leblond & Walker* (1956) *Bartman* (1961) and *Dustin & Bartman* (1961) discussed this question, and *Edwards & Klein* (1961) maintained that the hypothesis that all cells in a species have the same mitotic duration has little or no evidence to its support *Elgjo & Eliassen* (1963) have confirmed earlier observations of considerably increased mitotic counts in hibernating hedgehogs Their interpretation of this observation was that the increased number of mitoses is due to an increased mitotic duration caused by the lowered temperature and not to an elevated mitotic rate There is no reason to believe that the mechanism of mitosis differs from other physiological processes which all are slowed down during hibernation

The present experiments were instigated to study the significance of the variations in the number of mitoses during night and day under different conditions, taking into consideration even the mitotic *duration*

This latter parameter was studied by the colcemid technique according to *Leblond & Stevens* (1948) This provides an indirect method for an estimation of the mitotic duration Colcemid in proper doses arrests the mitoses without producing any observable changes in the non-dividing cells (*Schar, Loustalot & Gross* 1954) Colcemid permits cells to enter into mitosis, but does not stimulate cells to divide (*Dustin & Bartman* 1961) Colcemid arrests the mitoses in early metaphase The dividing cells in ana and telophase at the time of injection of the drug, however, fulfill their mitosis (*Dustin* 1959) If, therefore, the mitotic rate is low and the mitotic duration is longer than the observation period the number of mitotic figures observed after the colcemid injection may be about the same as, or even lower than the number observed without the influence of colcemid

EXPERIMENTS

In the present experiments we have used the strain of hairless mice (hrhr) of the institute These animals are vigorous and consume more food than mice with hair [illegible] ... outine ... 0 and ... start ... the afternoon the ... rest in a cluster, and are usually quiet till about midnight From then they become more and more active till about six or eight o clock in the morning Then they usually again have a period of rest till about 10 a m

The animals used in each experiment have been of the same age and in each experimental group there has been an [illegible] exper [illegible] same [illegible] whic [illegible]

Acta path et microbiol scandinav 64, 176 184 1965

Institutt for Generell og Eksperimentell Patologi Universitetet i Oslo
Head Leiv Kreyberg, M D

INFLUENCE OF STRESS ON THE DIURNAL RHYTHM IN THE MITOTIC ACTIVITY IN THE EPIDERMIS OF HAIRLESS MICE

By

LEIV KREYBERG, ARNE EVENSEN and OLAV HILMAR IVERSEN

Received 17 xi 64

Rhythmic variations in physiological processes are well known in plants and animals Such variations are often related to night and day and/or to rest and activity In 1903 *Maas* reported rhythmic variations also in mitotic count in different organs of cephalopods, and *Sabin* (1920) demonstrated periodic variations in the mitotic count in organs of chick embryos In 1922 *Kornfeld* showed that variations in the mitotic count in the corneal epithelium of salamanders were related to feeding and starvation *Bullough* (1946) demonstrated that variations in the epidermal mitotic count in adult female mice were related to the estrous cycle

Diurnal rhythmic variations in the mitotic count were first shown by *Droogleever Fortuyn-van Leyden* (1916) in the cells of the mesenterium and the corneal epithelium of the cat Diurnal variations in the mitotic count in the epidermis and in other organs have also, among others, been demonstrated by *Cooper & Schiff* (1938), *Cooper & Franklin* (1940), *Blumenfeld* (1942), *Bullough* (1948 a), *Bertalanffy* (1960), *Vasama* (1961), *Evensen* (1963) *Bullough* (1948 b) and *Bullough & Laurence* (1962) found that variations in the mitotic count in the ear epithelium of mice were closely related to muscular activity and rest Regular diurnal mitotic cycles can thus be found only in animal colonies having a higher standardized pattern of activity and sleep

At this point it is essential to emphasize that the observations mentioned above are based upon registration of mitosis seen and counted in the tissues, whichever the designation is, "number of mitoses', "mitotic count", "mitotic index", or others The validity of the conclusions drawn depends, however, upon one important factor *viz* that the mean duration of the mitoses always is the same in the same type of cells

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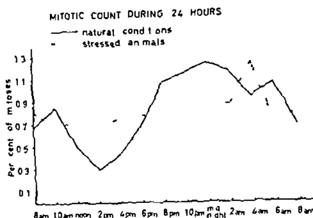


Fig 1

Variations in mitotic count in the back skin epidermis of hairless mice during the 24 hours The unbroken curve shows the 24 hours rhythm in untreated animals the broken curve shows the variations in animals exposed to acoustic stress

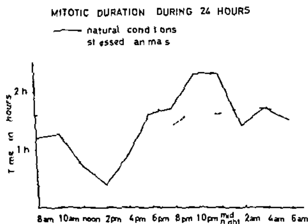


Fig 2

Variat ons in mitotic duration in the back skin epidermis of hairless mice during the 24 hours The unbroken curve shows the 24 hours rhythm in untreated animals the *broken curve shows* the variations in animals exposed to acoustic stress

The findings are recorded in Table 2 and Fig 2 It is seen that the *pattern* of the diurnal changes in the mitotic duration is very similar to the *pattern* of the changes in the diurnal count, both with their low points at *2 p m* and both with their flattened peaks on both sides of midnight

These observations strongly indicate that the diurnal rhythm in the mitotic counts *actually is caused by changes in the mitotic duration* It is easily seen from Figs 1 and 2 that the *mitotic rate* i e per cent of dividing cells per hour, seems to be almost constant during the 24 hours

Eperiments Nos 1 and 3 have been performed twice (in January and in May) The results, which did not show any significant difference, have been incorporated in one table and one curve for each experiment The experiments concerning mitotic duration have been performed parallel to the experiments concerning rhythms in mitotic count

The animals were killed by neck fracture The skin was immediately flayed off and immersed in a four per cent solution of neutralized formalin until the next day A skin piece, measuring approximately 1 by 2 cm was excised symmetrically from the midline on both sides of the back skin Histological sections of the skin specimens were stained with haematoxylin eosin Mitoses were scored among 2000 nucleated epidermal cells The mitotic count used in calculations of the mitotic duration is the arithmetic middle of the mitotic count done in the beginning and in the end of the observation period of two hours

Experiment I The Natural Diurnal Rhythm

1 *Mitotic count* Eight animals living under standard conditions were killed every two hours during a 24-hour-period The mitotic counts are shown in Table 1 and Fig 1

TABLE 1
Experiment No 1
Mitotic Count during the 24 Hours
Natural Conditions

Number of animals	Time when killed	Mitoses (mean per cent)
8	8 a m	0 68 ± 0 08
8	10 a m	0 85 ± 0 11
8	noon	0 50 ± 0 03
8	2 p m	0 29 ± 0 06
8	4 p m	0 43 ± 0 09
8	6 p m	0 68 ± 0 04
8	8 p m	1 07 ± 0 08
8	10 p m	1 10 ± 0 10
8	midnight	1 20 ± 0 10
8	2 a m	1 11 ± 0 05
8	4 a m	0 94 ± 0 08
8	6 a m	1 05 ± 0 05

The findings repeat the conventional 24-hour-rhythm, showing a fall from late morning until the feeding excitement has terminated Then a gradual increase begins with a slightly irregular, flattened peak from eight o'clock in the evening till eight o'clock in the morning

2 *Mitotic duration* Four groups of twelve animals under similar standard conditions were used The groups were injected with 0 1 mg of colcemid in 1 25 ml of saline solution (approximately 5 mg/kg body weight) with six hours intervals, the first group at 8 a m , the second group at 2 p m , the third group at 8 p m , and the last group at 2 a m Four animals from each group were killed every two hours after the injection of colcemid, namely two, four and six hours after the injection In this way four colcemid injected animals were killed every two hours during the 24-hour-period, and each animal had been under the influence of colcemid from two to six hours when sacrificed

watch dial 30 minutes apart When the long hand of the watch reached each of these connecting points, the electric bell started ringing for 30 seconds The ringing started in the morning (at 6 a m) and continued intermittently for 24 hours The ringing was very sharp, and the animals were considerably upset, especially during the first half of the experiment, some running about, others nesting in a corner In the course of the day the remaining animals seemed gradually to adapt themselves to the extraordinary noise,—or being exhausted, and after midnight the animals nested for sleep as usual, in spite of the repeated ringings

Every two hours a group of eight animals were killed and the mitosis in the back skin epidermis counted

1 *Mitotic count* The number of mitoses counted is recorded in Table 3 and in Fig 1 The diurnal count is definitely changed The fall in number of mitoses observed with a minimum about 2 p m is eliminated and for more than twelve hours the count is nearly constant and elevated as compared to the normal controls Only late at night, when the animals through their external behaviour seem to be sufficiently adapted to, or exhausted by, the extraordinary noise, a belated increase takes place

TABLE 4

Experiment No 4
Mitotic Duration during the 24 Hours
Stressed Animals

Time interval		Mitoses without colcemid arithmetic middle of two observations (from table 3)	Arrested mitoses (mean per cent) colcemid	Average mitotic duration
8 a m	10 a m	0 74	0 95 ± 0 03	1 6 h
10 a m	noon	0 70	0 81 ± 0 09	1 7 h
noon	2 p m	0 69	0 75 ± 0 08	1 7 h
2 p m	4 p m	0 72	0 85 ± 0 06	1 7 h
4 p m	6 p m	0 78	0 81 ± 0 07	1 9 h
6 p m	8 p m	0 77	0 78 ± 0 09	2 0 h or more
8 p m	10 p m	0 76	1 10 ± 0 07	1 4 h
10 p m	midnight	0 84	1 00 ± 0 09	1 7 h
midnight	2 a m	0 89	1 14 ± 0 07	1 6 h
2 a m	4 a m	1 06	1 25 ± 0 06	1 7 h
4 a m	6 a m	0 97	0 99 ± 0 08	1 7 h
6 a m	8 a m	0 75	1 11 ± 0 09	1 4 h

2 *Mitotic duration* Forty eight animals were exposed to the ringing of the electric bell as described in Experiment II, 1 Groups of twelve animals were injected with 0 1 mg of colcemid in 0 25 ml of saline solution at the same hours as in Experiment 1, 2, and the animals were killed every two hours during the 24 hours The results are shown in

TABLE 2

Experiment No 2
Mitotic Duration during the 24 Hours
Natural Conditions

Time interval	Mitoses without colcemid arithmetic middle of two observations (from table 1)	Arrested mitoses (mean per cent) colcemid	Average mitotic duration
8 a m – 10 p m	0.76	1.25 ± 0.16	1.2 h
10 a m – noon	0.67	1.08 ± 0.20	1.3 h
noon – 2 p m	0.39	1.00 ± 0.10	0.7 h
2 p m – 4 p m	0.36	1.63 ± 0.15	0.4 h
4 p m – 6 p m	0.55	1.20 ± 0.14	0.9 h
6 p m – 8 p m	0.87	1.06 ± 0.09	1.6 h
8 p m – 10 p m	1.11	1.30 ± 0.15	1.7 h
10 p m – midnight	1.15	1.16 ± 0.14	2.0 h or more
midnight – 2 a m	1.16	1.17 ± 0.15	2.0 h or more
2 a m – 4 a m	1.05	1.45 ± 0.18	1.4 h
4 a m – 6 a m	0.99	1.20 ± 0.09	1.7 h
6 a m – 8 a m	0.86	1.10 ± 0.08	1.5 h

TABLE 3

Experiment No 3
Mitotic Count during the 24 Hours
Stressed Animals

Number of animals	Time when killed	Mitoses (mean per cent)
8	8 a m	0.81 ± 0.12
7	10 a m	0.68 ± 0.10
8	noon	0.72 ± 0.11
8	2 p m	0.67 ± 0.10
8	4 p m	0.77 ± 0.12
8	6 p m	0.79 ± 0.09
8	8 p m	0.75 ± 0.09
8	10 p m	0.78 ± 0.08
8	midnight	0.91 ± 0.19
8	2 a m	0.87 ± 0.12
8	4 a m	1.25 ± 0.13
8	6 a m	0.69 ± 0.08

These findings made it natural to start another series of experiments namely with the aim of examining the mitotic manifestations during conditions where the natural rhythm is broken through stress

Experiment II The Diurnal Rhythm During Stress

Ninety-six animals were moved, in their own cages into an isolated room A strong electric bell placed very near the cages was connected to the long hand of a watch Two connecting points were placed on the

stressing stimuli and that the mitotic count alone cannot be used for an estimate of the mitotic rate

SUMMARY

The diurnal variations in the epidermal mitotic counts in hairless mice seem to be caused by changes in the mitotic duration, the mitotic rate being fairly constant This holds good for animals under natural conditions as well as for animals when the diurnal variations have been markedly changed by acoustic stress The influence of adrenal hormones on the kinetics of the epidermal cell proliferation is discussed

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Table 4 and in Fig 2 and demonstrate that the rhythmic variations in the mitotic duration seen in the animals under natural conditions have been smoothened out to a great extent

Again, the similarity of the curves expressing the mitotic count and the mitotic duration is striking, and again the experiments demonstrate that even in heavily stressed animals the *mitotic rate* is probably un changed during the 24 hours

DISCUSSION

Experiment no 1 demonstrates that the mitotic count is lowest when the animals are most active, about the time of feeding and handling and that the mitotic count increases when the animals are resting and sleep ing Experiment no 2 shows that the mitotic duration varies almost parallel with the variations in the mitotic count—the more muscular activity, the shorter the mitotic duration The mitotic rate, however calculated from the observations in experiments nos 1 and 2 does not show similar variations during the 24 hours It is well established that the amount of adrenaline in the blood varies during the 24 hours, the level being raised during muscular activity and lowered during rest and sleep *Evensen* (1964) and *Evensen & Heldaas* (1964) have demonstrated that adrenaline may shorten the mitotic duration in epidermis both *in vivo* and *in vitro* The profile of the curve of experiment no 2 may there fore be interpreted as caused by the variations in the adrenaline blood level during activity and rest

Selye (1950) maintains that nervous stimuli, among them intense light and sound, provoke the "alarm reaction" This reaction provokes an immediate discharge of adrenaline from the adrenal marrow into the blood Somewhat more slowly, but still during the first hour of the "alarm reaction", the adrenal cortex begins to discharge its stores of corticoid hormones During the "alarm reaction" the animals are thus for the first hour mostly under influence of adrenaline, later, when the stores of adrenaline are emptied, under the influence of corticoid hor mones

In experiments nos 3 and 4 the animals had been stressed by the ring ing bell for two hours when the first groups were killed, and the an imals had then been under the influence of hormones, both from the adrenal marrow and from the adrenal cortex

Experiments nos 3 and 4 demonstrate that heavy stressing stimuli may provoke disturbances in the epidermal cell proliferation, probably by changes in the amount of adrenal hormones in the blood

It can be concluded that the mitotic duration in epidermis is increased during rest and sleep, and is shortened during muscular activity, and that the well known diurnal variations in the mitotic count in the epi dermis may be caused by variations in the mitotic duration It can also be concluded that the epidermal cell proliferation is influenced by

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Institutt for generell og eksperimentell patologi Rikshospitalet Oslo
(Head Professor Olav Hilmar Iversen M D)

CELL POPULATION KINETICS IN MOUSE EPIDERMIS AFTER REPEATED APPLICATIONS OF 20 METHYLCHOLANTHRENE

By

KJELL M ELGJO and ØYVIND SKJÆGGESTAD

Received 24 xi 64

During the last few years it has become increasingly evident that malignant tumours do not necessarily grow faster than normal tissues The present situation has been summed up by *Kaplan* in 1963, stating that "Years ago this (unrestricted growth) was associated in our minds with the idea that each cancer cell grew faster than do normal cells However more careful analysis clearly establishes that this is not the case Indeed we now realize that many cancer cells divide less often than some normal cells "

In most studies the interest has been focussed on the cancer cell itself and its growth parameters Little attention has been paid to the growth regulation within cell populations during the development from normal conditions to malignant tumours although several techniques at present are available for such studies All methods, however, have originally been worked out on normal tissues Accordingly, one of the main difficulties is that we don t know if the effect of the agents used in the different methods is the same in neoplastic and in normal tissue In the present study two independent methods have therefore been applied simultaneously to evaluate the growth of the epidermal cell population in the hairless mouse during methylcholanthrene-induced carcinogenesis

Method 1 is the conventional Colcemid method which has been extensively discussed by several authors (*Eigsti & Dustin* 1955, *Leblond* 1959 *Hooper* 1961) Method 2 has been adapted by ... [illegible]

[illegible] ... slope of this disappearance curve the turnover time of the differentiating cells with their corresponding horny layer as well as the mean generation time of the basal cells can be estimated The turn over time in the normal epidermis is about 4–4½

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benzene to the interscapular area by means of a Pasteur pipette The treatment was continued for 4 weeks (12 applications in all) then stopped The mice were divided into two groups and each group treated according to one of the following methods

Method 1

Groups of eight mice were studied 2 7 12 and 16 days after the last treatment Four mice in each group were injected with 0 1 mg of Colcemid (CIBA) intraperitoneally 4 hours before they were sacrificed The skin was flayed off and fixed in

plete their mitoses per unit time can be found by the equation mitotic rate = (mitotic count)/(mitotic duration)

In addition the thickness of the epidermis was measured by means of an ocular micrometer in five randomly chosen fields in all the animals

Method 2

One day after the last application of methylcholanthrene 50 μc H^3 thymidine was injected intraperitoneally and the mice were killed 1 3 6 8 and 10 days later The skin was flayed off and an epidermal specimen separated by means of a special ker

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dis

Qu collection was performed and the H^3 activity was expressed as count per mg dry epidermis

RESULTS

Method 1

At the time when treatment is discontinued the mitotic count is increased to about 1 7 times the normal value, and the mitotic duration to about 1 6 times the normal value The mitotic rate is thus slightly greater than normal When the treatment is stopped both rate and count increase temporarily, then fall gradually approaching the normal values after about 2 weeks The mitotic duration immediately decreases and is within the normal range after two weeks (Table 1 and Fig 2)

TABLE 1

Changes in Epidermal Mitotic Count Duration and Rate during the First 16 Days Subsequent to 4 Weeks Repeated Applications of 20 methylcholanthrene

Time after last application of methylcholanthrene (days)	Mitotic count per 6mm	Mitotic duration (h)	Mitotic rate per 6mm
2	16 50 ± 3 00	1 58	10 42
7	21 00 ± 4 60	1 49	14 01
12	15 75 ± 3 00	1 49	10 55
16	11.50 ± 3 87	1 11	10.39

The mitotic counts represent the means of groups of 4 mice Forty fields (magn 1000 ×) were counted in each specimen The mitotic count duration and rate are the means of observations on 8 mice 4 killed before and 4 after a delay of 4 h after injection of Colcemid

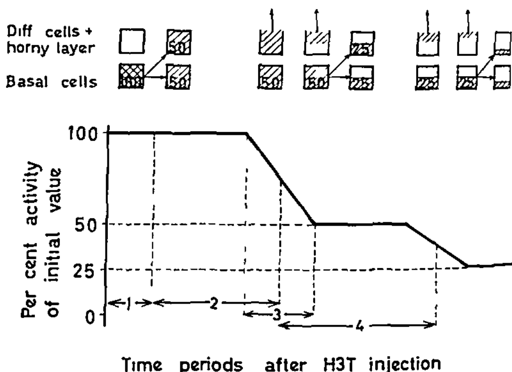


Fig 1

Theoretical scheme of the amount of H^3 DNA activity assumed to be in the epidermal cell population at different time intervals after a single injection of H^3 thymidine The symbols on the abscissa designate

1 Time period from labelling (injection) until the cells have divided
2 Average 'turn over time" of differentiating cells together with horny layer
3 Variations in the 'turn over-time'
4 'Mean generation time' of the basal cells

days, while the mean generation time of the basal cells is 5–5½ days Method 2 cannot, however, be used without reservation in irregular hyperplastic epidermis because mitoses then appear even in the differentiating layer Further, it is uncertain if each mitosis in this hyperplastic state always produces one differentiating and one cell capable of further division, as is the case in the normal epidermis Hyperplastic epidermis is thus no longer an "ideal cell population" Nevertheless, the slope of the H^3-disappearance curve gives information about the mean generation time of the dividing cells, *i e*, the rate of cell renewal, especially when the curve is compared with that obtained from a normal epidermal cell population

METHODS AND MATERIALS

Procedures Common to both Methods

Hairless mice (hr/hr) about 3 months old of both sexes were treated three times weekly by applying 4–5 drops of a 0 6 per cent solution of methylcholanthrene in

benzene to the interscapular area by means of a Pasteur pipette The treatment was continued for 4 weeks (12 applications in all), then stopped The mice were divided into two groups and each group treated according to one of the following methods

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THEORETICAL H3-DNA DISAPPEARANCE CURVE

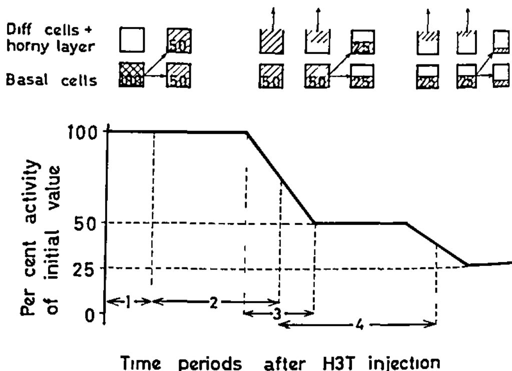


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the horny layer in these populations is about the same From the third day the activity decreases in both populations but the fall is steeper in the hyperplastic epidermis From the slope of these curves the mean generation time of the basal cells can be calculated In the hyperplastic epidermis the mean generation time is 4 days, in the normal epidermis 5 35 days The difference between the steepness of the two curves is significant ($P < 0\ 05$) and the cell renewal rate is thus significantly higher in the hyperplastic epidermis If the normal epidermal cell renewal rate is set at 1 0 the cell renewal rate in the hyperplastic epidermis is about 1 3 (5 35 4 00)

TABLE 2

H^3 Activity in Dried Hyperplastic Epidermis at Different Time Intervals after Intraperitoneal Injection of H^3 Thymidine

Days after injection	No of mice	Activity Mean	$SD_{(\bar{x})n}$
1	6	160 1	12 2
3	7	156 0	10 5
6	6	89 4	6 7
8	7	77 1	5 0
10	8	54 5	2 6

The H^3 DNA activity in the hyperplastic epidermal specimen at different intervals after the injections of 50 µc H^3 thymidine

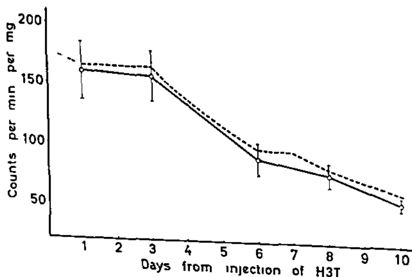


Fig 3

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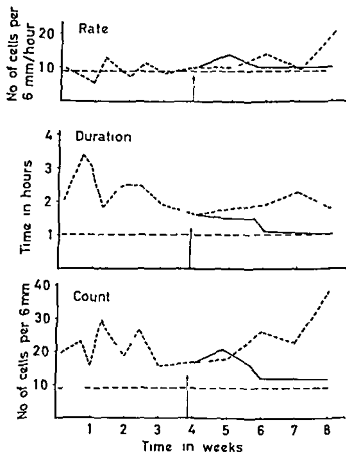


Fig 2

Variations in mitotic count duration and rate after repeated applications of 20 methylcholanthrene to the epidermis

The values of the mitotic count, duration and rate after discontinued treatment are indicated by (————) the values during continuous treatment by (-- --) An *arrow indicates the time of the last application of 20 methylcholanthrene* in the present study

If the treatment is continued, however, both the mitotic count, the duration, and the rate tend to increase

In Fig 2 the mitotic count, duration, and rate during the time prior to the period of the present investigation have been indicated, as well as the values of these parameters during continued treatment after 4 weeks These values are taken from an earlier paper by one of the present authors (*Elgjo* 1964)

Method 2

In Table 2 the radioactivity in the hyperplastic epidermis is shown at different intervals after the injection of H^3-thymidine In Fig 3 these values are compared with those found in the normal epidermis It is seen that in both cell populations the activity is constant the first three days, indicating that the turn over-time of the differentiating cells and

is slightly elevated During the first days after discontinuing the treatment the mitotic rate shows an additional and transient increase, which is followed by a gradual decrease Thus, during the period in which the loss of activity from the epidermis is measured the mitotic rate estimated by means of the Colcemid method is increased within a range that is compatible with the increase found by means of method 2 Accordingly, as both methods reveal the same trends of the different growth parameters the following joint conclusions seem to be justified

1 The pronounced increase in the mitotic count found after repeated applications of methylcholanthrene is mainly due to a prolonged mitotic duration and only partly caused by an increased mitotic rate

2 After four weeks' repeated applications of methylcholanthrene the mitotic rate is only moderately raised, while the turn-over-time of the d fferent ...

nected with the presence of this compound, as all these parameters tend to approach the normal range when the treatment is stopped

4 At the period of hyperplasia studied in the present investigation there seems to be a disequilibrium between the cell renewal and the cell loss Both methods reveal a moderately increased rate of cell renewal The turn-over time however is not decreased This indicates that the hyperplasia may be explained as the result of a moderately increased rate of cell renewal without a correspondingly increased cell loss

SUMMARY

The kinetics of the epidermal cell population after repeated applications of 20 methylcholanthrene has been investigated by two independent methods The mitotic duration and the mitotic rate have been estimated by means of the conventional Colcemid technique The mean generation time of the dividing cells and the turn over-time of the differentiating cells have been evaluated by means of a technique that is ...
rom epidermal cell nuclei
parameters estimated by
the cellular proliferation
... indicating that the hyperplasia found after repeated applications of 20 methylcholanthrene may be explained by a slightly increased mitotic rate without a corresponding increase of the cell loss

REFERENCES

Berg ...

DISCUSSION

The pathogenesis of the hyperplasia found after application of carcinogenic and non-carcinogenic agents has been a controversial subject and several explanations have been offered Thus, *Glucksmann* (1945) and *Berg* (1948) have interpreted the hyperplasia found after painting with benzpyrene as a result of a mitotic stimulation Another explanation has been given by *Berenblum* (1954) suggesting a disequilibrium of the cell growth with exponential growth instead of linear growth *Setälä et al* (1962), however, has paid much attention to the discrepancy between the number of mitoses and the hyperplasia found after painting with carcinogenic agents These investigators also observed a different growth rate in "benign" and "malignant" hyperplasia suggesting a 'delayed maturation" of the epidermal cells in "malignant" hyperplasia *Iversen & Evensen* (1962) have discussed this problem in relation to the early changes in the epidermis of the hairless mice after a single application of carcinogenic and non-carcinogenic agents They also applied several methods to the same investigation, *i e*, a tetrazolium reduction method, autoradiography, and the Colcemid technique Their results indicate that the hyperplasia following a single application can be well explained as a regenerative process, *i e*, a shift to a population containing a higher proportion of young cells Their results have later been confirmed by *Skjæggestad* (1964) using the technique called "Method 2" in the present paper

TABLE 3

Changes in the Thickness of Epidermis during a Period of 16 Days Following 4 Weeks with Repeated Applications of 20 methylcholanthrene

Time after last application of MCA (days)	Average thickness of epidermis (μ)
2	38 0 ± 3 8
7	38 5 ± 7 1
12	33 6 ± 2 8
16	32 6 ± 6 1

The values represent the means of measurements on groups of 8 mice Each specimen was measured at 5 different places chosen at random

The calculation of the mean generation time in the present study (method 2) is made under the assumption that each dividing basal cell produces one differentiating cell and one new basal cell, in other words, that the growth is linear As is seen from Table 3 the thickness of the epidermis is not constant after the applications have been stopped The epidermis becomes gradually thinner, indicating that the cell population decreases in number In consequence, the calculated cell renewal rate is a maximum value The cell renewal rate is most probably increased during this period, but not more than 1 3 times the normal

The findings by the Colcemid method confirm the observations mentioned above At the time when the treatment is stopped the mitotic rate

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INFLUENCE OF THE THYMUS ON NORMAL AND THYROXIN STIMULATED REGENERATION OF LYMPHATIC TISSUE AFTER STEROID INDUCED INVOLUTION IN GUINEA PIGS

By

ULF ERNSTRÖM and LARS GYLLENSTEN

Received 5.xii.64

In several laboratory rodents cellular or humoral thymus factors are necessary for the normal growth and differentiation of the lymphatic tissue (including the appearance of immunologic competence) during the early postnatal stages (ref. see *Miller et al.* 1962, *Martinez et al.* 1964). In older animals thymectomy has less or no significant influence and this also applies to animals, e.g. guinea pigs, which are more mature at birth than rats or mice. This is possibly because in these animals the thymus has already exerted its influence on the lymphatic organs.

Even in mature animals quantitative studies may disclose some impairment of immunologic reactions after thymectomy (*Fichtelius et al.* 1961, *Å ar* 1963). In adult rats and mice histological regeneration of lymphatic organs and recovery of immunological reactivity following X irradiation depend on the thymus—thymectomy causing a retardation of restitution (*Globerson et al.* 1962, *Miller* 1962, *Auerbach* 1963, *Miller et al.* 1963, *Cross et al.* 1964, *Globerson & Feldman* 1964). Most probably one or seve[illegible] cells of the thym[illegible] spleen and lym[illegible] livered from the [illegible] reaching the mentioned lymphatic organs and [illegible] populating them or inducing them to produce e.g. lymphocytes and plasma cells (ref. *Fichtelius* 1960, *Miller* 1964, *Osoba & Miller* 1964).

As early as 1956, before the recent era of thymus research, *Gregoire & Duchateau* found that implants of irradiated thymus tissue composed of epithelial cells but practically devoid of lymphocytes stimulated lymphopoiesis in adjacent lymph nodes. *Harris & Ford* (1964) and *Harris et al.* (1964) using chromosome markers in graft and parabiosis experiments have provided evidence of another mechanism, i.e. that

The expences of the investigation were partly defrayed by a grant from the Swedish Cancer Society.

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Figsti O I ° ogy and Chemistry

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The aim of the present investigation was to study the significance of the thymus for normal regeneration and thyroxin-stimulated regeneration of the lymphatic tissue after steroid induced involution It is part of a larger project on the hormonal control of the thymo lymphatic tissue

MATERIAL AND METHODS

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roid induced involution has been shown to have reached or passed its maximum and

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In after sham operation or thymectomy The animals were killed by a blow on the neck and the following lymphatic organs were dissected out quantitatively and weighed in the fresh state thymus cervical lymph nodes scapular lymph nodes inguinal lymph nodes and spleen The technique of the operations as well as the anatomy and dissection of the organs have been described previously (*Gyllensten* 1953) All organ weights were calculated as relative weights (mg/100 g of body weight) Such a calculation is legitimate only if certain criteria are fulfilled regarding the regression line and the correlation between organ weight and body weight (*Gyllensten* 1953 *Ingervall & Carlström* 1963) The legitimacy of the method was confirmed for the present material

RESULTS

The relative weight of the thymo lymphatic organs in all groups of animals is shown in Fig 1 No differences in mean body weight were demonstrated

For *control animals*, Group SC the relative weight of the organs is recorded in Table 1 The standard errors and, consequently the coefficients of variation, are comparatively high (cf *Gyllensten* 1953) This depends on the fact, that the animals consisted of several subgroups, which were procured on different occasions, a variation being present between these subgroups On each occasion, animals were taken for all experimental groups (the main groups SC, SU, TU, SUT and TUT), and the differences between the groups were computed separately on each occasion The statistical significance of the differences was calculated by Student's *t* test and finally, combined *t* values for all groups were computed using the formula

$$u = \frac{\Sigma t}{\sqrt{\Sigma \frac{df}{df - 2}}}$$

where *t* and *df* are the corresponding *t* values and degrees of freedom of the separate samples

the thymus receives lymphoid cells which are produced in other parts of the haemopoietic system (bone marrow?) and instructs these cells so that, if stimulated by antigens, they become competent to proliferate and differentiate in the spleen and lymph nodes

Thymus, *antigens and some hormones (steroids, thyroxin)* are known to influence the normal growth and mass of the spleen, lymph nodes and scattered lymphatic tissue In contrast, the factors which regulate the activity of the thymus (lymphocytopoiesis, lymphocytolysis, and release of cells or other substances) have been explored to a limited extent It is not difficult to produce thymus involution, *e g* by means of steroids or X-irradiation Hyperplasia or hyperactivity of the thymus can be induced by adrenalectomy or castration, causing a fall in steroid concentration and decreased steroid inhibition of the thymus but not primary stimulation of it In young animals compared to old ones (*Kindred* 1940, *Andreasen & Christensen* 1949), and in regeneration after experimental involution (*Ishidate & Metcalf* 1963), increased mitotic frequency is observed in the thymus Little else is known about stimulation of the thymus, and experimental procedures for stimulation are scanty, antigens being without obvious effect (*Miller* 1964) The best-established mechanism for stimulation of thymus growth is administration of thyroid hormones (ref *Gregoire* 1942, *Gyllensten* 1953, *Lundin* 1958, *Ernstrom & Gyllensten* 1959), either directly by injection of these hormones, or indirectly by injection of thyrotrophic hormone Part but not all of this thyroid effect on thymus growth may be due to increased catabolism of steroids (*Marder* 1951, *Lundin* 1958, *Dougherty et al* 1962)

Compensatory growth of thymo-lymphatic tissues after its reduction *may be partly directed by slight hyperactivity of the thyroid gland* (*Gyllensten* 1953) In addition to this homeostatic reaction by the thyroid, the lymphatic tissue has been found, during regeneration (after steroid-induced involution in guinea-pigs), to react by greater proliferation on thyroid stimulation than does normal lymphatic tissue (*Barnholdt-von Euler et al* 1959, *Gyllensten* 1962 b)

The aforementioned facts suggest that the thymus and the thyroid might cooperate in promoting growth and regeneration of the rest of the lymphatic tissue (*lymph nodes, spleen and scattered lymphatic tissue*) It is not known how the thyroid and the thymus influence the lymphatic tissue—they may act in a similar or synergistic way, or they may act separately, through different mechanisms In recent investigations by *Ernstrom* (1965) on the influence of thyroid hormone in normal and thymectomized young guinea pigs, it has been demonstrated that thyrogenous lymphatic hyperplasia is potentiated by the presence of the thymus, but is not exclusively dependent on it If there is no synergism, local differences might exist in *the involuted lymphatic tissue*, which cause it to react more intensely to administration of thyroid hormone (*Barnholdt-von Euler et al* 1959, *Gyllensten* 1962)

after steroid administration was slower than in animals with intact thymus (Figs 1 and 2, Table 2) In animals of group TU as well, the lymph nodes of the extremities lagged behind the cervical nodes (Fig 2)

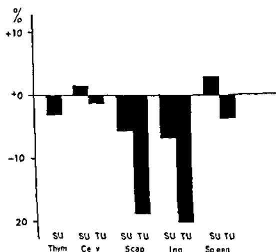


Fig 2

Percentage change in relative organ weight in animals given steroid after sham operation (SU) or thymectomy (TU) Greater involution of the lymph nodes and spleen is demonstrated in the latter The scapular and inguinal lymph nodes are more involuted than the cervical nodes

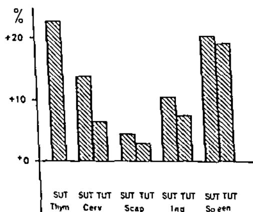


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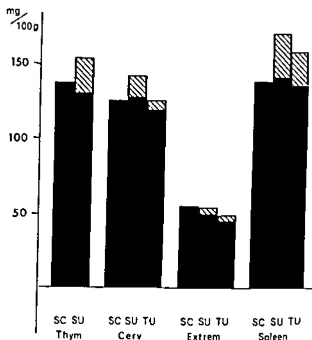


Fig 1

Relative weight (mg/100 g body weight) of the thymus, cervical lymph nodes lymph nodes of the extremities, and the spleen in the controls (SC) and in the animals given steroid after sham operation (SU) or thymectomy (TU) black columns Persistent involution is demonstrated in the last-mentioned, but no consistent involution in the sham operated animals

The hatched columns show the influence of thyroxin on sham operated steroid treated animals and on thymectomized steroid-treated animals, respectively Increase in weight of organs is demonstrated after thyroxin administration

TABLE 1

Relative Weight (mg/100 g Body Weight) of the Thymo Lymphatic Organs in Controls (Group SC) — Mean ± Standard Error

Lymph nodes				Thymus	Spleen	n
Cervical	Scapular	Inguinal	Total lymph node mass			
126 5 ±6 74	28 2 ±1 43	27 4 ±1 33	182 1 ±8 09	136 7 ±5 25	138 6 ±3 86	46

As apparent from Fig 1, slight *thymo-lymphatic involution* persisted in the thymus and the lymph nodes of the extremities 8 days after administration of corticosteroid, but not in the cervical lymph nodes or spleen This confirms the results of *Gyllensten* (1962 a), *i e*, that after steroid-induced involution, regeneration of the cervical lymph nodes is more rapid than that of the extremity nodes Statistical analysis demonstrated no significant differences between organ weights in controls and in steroid-treated animals (Groups SC and SU, respectively) In thymectomized guinea pigs given steroid (Group TU), on the contrary, significant involution persisted This implies that regeneration

The *effect of thyroxin* on the thymo-lymphatic organs after steroid induced involution in animals *with intact thymus and in thymectomized* animals is demonstrated in Figs 1 and 3 In all groups thyroxin caused an increase in weight of the lymphatic organs *The stimulating effect* was greater in animals with intact thymus than in thymectomized animals The differences and statistical evaluations are recorded *in Table* 3 In animals with intact thymus thyroxin produced a highly significant increase in the relative weight of the spleen a significant increase in the weight of the thymus and an almost significant increase in the weight of the lymph nodes In thymectomized animals the differences between thyroxin treated and not thyroxin treated animals were significant as regards the spleen but not as regards the lymph nodes

The results can be summarized in two main statements *Firstly* thymectomy produced a decrease in spontaneous and thyroxin stimulated regeneration of the lymphatic organs after steroid induced involution *Secondly* thyroxin produced an increase in regeneration of the lymphatic organs after steroid induced involution more pronounced in animals with intact thymus These main statements can be illustrated by calculation of the differences between the weight of the lymphatic organs in thymectomized and in non thymectomized animals (irrespective of thyroxin administration) as well as of the corresponding differences between animals given thyroxin and not given thyroxin (irrespective of thymectomy) Such calculations are summarized in Table 4 They show that *thymectomy* caused a statistically significant *decrease* in weight of regenerating lymphatic tissue whereas *thyroxin* caused a statistically significant *increase*

TABLE 4

S ... *iphatic in (Ir*

		Cervical lymph nodes	Lymph nodes of extremities	Total lymph node mass	Spleen
Decreased organ weight after thymectomy	t	1 76	2 71	2 28	2 07
	p		$<0 01$	$<0 05$	$<0 05$
Increased organ weight after thyroxin	t	2 93	1 12	2 01	4 48
	p	$<0 01$		$<0 05$	$<0 001$

DISCUSSION

The present findings show that the thymus is necessary for normal regeneration of the lymphatic tissue after steroid induced involution and for the full effect of thyroxin stimulation during this regeneration Apparently regeneration occurs even after thymectomy and this also applies to the stimulating effect of thyroxin administration during regeneration Although minute amounts of thymus tissue persist after

TABLE 2

Mean Differences between Relative Weight (mg/100 g Body Weight) of the Lymphatic Organs in Controls (Group SC) and Thymectomized Animals after Steroid Induced Involution (Group TU) — Mean ± Standard Error Significance of Differences Calculated by Means of Combined t Values

	Cervical lymph nodes	Lymph nodes of extremities	Total lymph node mass	Spleen	df
Group SC Group TU	4.23 ± 10.08	10.20 ± 3.12	14.46 ± 11.62	6.95 ± 5.36	82
p	—	< 0.001	< 0.05	—	

TABLE 3

Mean Differences between Relative Weight (mg/100 g Body Weight) of the Thymolymphatic Organs in Animals Given Thyroxin during Regeneration after Steroid Induced Involution and those not Given Thyroxin Influence of Thymectomy on Thyroxin-Stimulated Regeneration

	Cervical lymph nodes	Lymph nodes of extremities	Total lymph node mass	Spleen	Thymus	df
Group SUT Group SU	16.53 ± 10.29	3.92 ± 3.16	20.44 ± 11.80	34.63 ± 9.86	18.41 ± 10.79	73
p	< 0.05	—	< 0.05	< 0.001	< 0.01	
Group TUT Group TU	4.22 ± 10.13	3.82 ± 2.87	8.07 ± 11.20	24.48 ± 6.83	—	68
p	—	—	—	< 0.01	—	

Group SUT sham operation steroid thyroxin Group SU sham operation steroid Group TUT thymectomy, steroid, thyroxin Group TU thymectomy steroid — Mean ± standard error, p values calculated after combination of t values in different subgroups

to produce and/or release the factor(s) which is (are) important for the growth of lymphatic tissue Increased regeneration of the thymus was in fact observed after thyroxin administration which might be an indication of functional hyperactivity

SUMMARY

The influence of thymectomy and thyroxin on the regeneration (organ weight) of the lymphatic tissue after steroid induced involution has been studied in young male guinea pigs The following observations are made

1 Thymectomy causes decreased regeneration of the lymph nodes and spleen

2 Thyroxin causes increased regeneration of the thymus, lymph nodes and spleen

3 Even in thymectomized animals thyroxin causes increased regeneration of the lymphatic organs but this stimulation is statistically significant only as regards the spleen Thyroxin has a greater stimulating influence on the regenerating lymphatic organs in animals with intact thymus than in thymectomized animals

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thymectomy, (*Gyllensten* 1953), no compensatory growth of these remnants has ever been observed, and the remnants are extremely small If they are regarded as insignificant from a functional point of view, the results demonstrate that the lymphatic organs are to some extent competent to react autonomously by regeneration after involution, and by growth after thyroxin administration These conclusions are supported by investigations of *Ernstrom* (1965), showing that thyroxin causes an increase in weight of the lymphatic tissue in guinea pigs shortly after thymectomy, but to a lesser extent than in intact guinea pigs *Ernstrom* also demonstrated that the thymus-dependence of the lymphatic organs during thyroxin-induced growth is time-dependent thus, one month after thymectomy, thyroxin failed to produce normal lymphatic hyperplasia It might be that some thymus factors (factor) (cellular and/or humoral) persist(s) for a period after thymectomy, and contribute(s) to growth of the lymphatic organs after involution

The results also demonstrate that, even after steroid-induced involution, the thymus is competent to deliver some factor(s) for regeneration of the spleen and lymph nodes This is in accordance with findings of *Gregoire & Duchâteau* (1956) and *Osoba & Miller* (1964), i e, that thymus tissue after roentgen-induced involution or after growth in diffusion chambers, respectively—when only reticular and epithelial cells remain—still retains the ability to stimulate the growth of lymphatic tissue

Accepted that thyroxin does not act on the lymphatic tissue exclusively through the thymus, a plausible mechanism for the direct action of thyroxin is that the hormone stimulates mitotic activity in the spleen and lymph nodes Stimulation of mitosis following thyroxin administration has been noted in various tissues and organs (ref *Gyllensten* 1962 b) Increased protein anabolism has also been reported after thyroxin (*Tata et al* 1962, *Michels et al* 1963) During steroid induced involution of lymphatic tissue and its subsequent regeneration, inhibition of mitosis has been observed during the early stages, and increased mitotic activity later (*Baker et al* 1951, *Dougherty et al* 1962) The thyroxin administration in the present study may, therefore, have acted as a stimulator of mitosis and associated protein anabolism during regeneration, when the lymphatic tissue is specially adapted for proliferation As described by *Dougherty et al* (1962) and *Gyllensten* (1962), lymphatic tissue is richer in "immature", basophilic stem cells after steroid-induced involution than is normal lymphatic tissue Such cells are generally regarded as characteristic components of proliferating tissue This may be why thyroxin seems to be a more potent stimulator of growth in involuted lymphatic tissue than in normal lymphatic tissue (*Barnholdt von Euler et al* 1959, *Gyllensten* 1962) As previously stated, the presence of the thymus is a prerequisite for the full effect of thyroxin on lymphatic regeneration

An additional possibility is that thyroxin also stimulates the thymus

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LOCATION OF RENIN (OR A RENIN-LIKE SUBSTANCE) IN THE SUBMAXILLARY GLANDS OF ALBINO MICE

By

JENS BING and POUL FARUP

Received 9 xii 64

The submaxillary glands of albino mice have been shown to contain a pressor substance, (*Werle et al* 1957) which according to studies by *Werle et al* (1960–61, 1962 and 1963), and *Turrian* (1960) seems to be an isoenzyme of renin, which splits angiotensinogen to a decapeptide identical with angiotensin I As a complement to these observations the present experiments were undertaken with the aim of studying the location of renin in the submaxillary gland

METHODS

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TABLE 1

Preparation	Number of animals	Body weight in g		Mean weight in mg of 1 gland	Renin (G. Dog Units g)				Granulation of striated ducts
					Glands		Kidneys		
		Mean	Range		U g	U 2 gl	U g	U 2 k	
274	6	6 5	(5 9 7 5)	12 5	0				0
276	5	6 5	(6 1– 7 0)	13 1	0				
277	4	6 9	(6 4 7 1)	11 5	0				
278	4	7 0	(6 2 8 2)	11 6	0				
283	5	8 0	(7 2 8 1)	17 5	0		25	3 0	0
285	3	8 0	(7 2 8 1)	21 3	0				
286	5	9 4	(8 9 9 6)	20 4	0		40	5 3	(+)
288	4	9 4	(8 9– 9 6)	21 6	0				
269	5	11 2	(11 0–11 6)	22 0	0		62	9 4	(+)
291	3	11 2	(11 0–11 6)	24 6	13	0 7			
292	5	13 2	(12 8 13 7)	25 8	8	0 4	50	8 6	+
294	1	13 2	(12 8 13 7)	28 2	5	0 3			
295	5	15 1	(14 9 15 3)	23 2	50	2 3	67	12 0	+
297	3	15 1	(14 9–15 3)	25 2	25	1 3			
298	5	16 3	(14 9–16 9)	25 4	375	17 0	50	10 0	+
300	3	16 3	(14 9–16 9)	25 4	500	25 0			
394	1	17 3		23 0	500	23 0	75	16 0	
324	5	18 2	(17 7–18 7)	27 0	610	33 0			+
326	3	18 2	(17 7 18 7)	25 5	750	38 0			
327	5	20 0	(19 1–21 0)	27 6	2500	116 0	50	12 0	++
329	3	20 0	(19 1–21 0)	26 9	625	34 0			
400	2	21 8	(21 5 22 0)	27 0	6000	324 0	100	24 0	
354	1	24 0		42 0	2100	177 0			
356	1	24 0		46 8	3200	300 0			
334	1	25 0		34 0	2800	190 0	75	24 0	++
382	1	25 0		38 0	20000	688 0	75	23 0	+++
355	1	26 0		68 0	4200	520 0			
332	1	30 0		49 0	10000	980 0	75	35 0	+++
386	1	40 0		81 0	8000	1300 0	75	38 0	

Relation between renin content and granulation of striated ducts in the submaxillary glands, and renin content of the kidneys during the post natal development of albino mice The renin values are given in Goldblatt Dog Units both as the concentration in *Units per g of tissue (U/g)*, and as the total amount in the two glands (U/2 gl) and in the two kidneys (U/2 k)

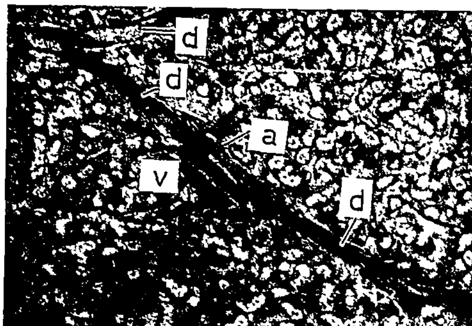


Fig 1

Freeze dried cryostat section immersed in drops of liquid paraffin The section of the gland, which contains numerous highly refractive granulated ducts is intersected by a bifurcated exeretory duct (d), close to which an arteriole (a) and a venule (v) are seen (Phase contrast × 100)

microscope at 400 times magnification in order to secure the 'purity' of the fractions Extraction of renin was performed with a Tris buffer (pH 7 5) in a micro homogenizer after determination of the size of the microdissected fractions by planimetry of enlarged microphotographs Neither the addition of liquid paraffin nor the treatment during the microdissection were found to change the renin content The animals were bled before removal of the glands so as to minimize the content of angiotensinase from erythrocytes

Some of the extracts still contained enough angiotensinase to affect the amount of angiotensin formed in the 10 minutes incubation It was thus necessary to determine the angiotensinase activity in parallel experiments and make a correction based on the effect found

Other parts of the glands were fixed in Helly's fluid and stained with Bowie "neutral dye" or iron haematoxylin

Determination of the area containing granules in microdissected fragments was performed with enlarged microphotographs which by means of an epidiascope giving a total magnification of 500 times were projected on paper on which the area was outlined and determined by planimetry By comparing the sum of the granulated area with the total area of the fragment percentage of granulated tissue could be determined

RESULTS

1 *Relation between Morphological Structure and Renin Content During the Post Natal Development of the Submaxillary Gland*

As it has been shown, that the rat submaxillary glands first reach the normal adult structure, when the animals are about 3 month old (*Jacoby & Leeson* 1959), and as *Werle & Vogel* (1960) did not find renin in the glands of mice before the animals were about 35 days old, mor-



Fig 3

In close connection with the ramification of an excretory duct (d) a bifurcating arteriole (a) and a venule (v) are seen The arteriole contains non granulated epithelioid cells (arrows) (Bowie stain × 600 in 3a and × 1200 in 3b)

nearly always located in arterioles placed close to the intralobular ducts (Fig 3a and b) while they were lacking in the periphery of the gland A granulation of the epithelioid cells similar to that found in the juxtaglomerular epithelioid cells of the kidneys was never observed The epithelioid cells in the glands further differ from those of the normal

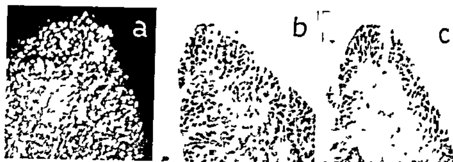


Fig 2
Sections of the submaxillary gland from a mouse, weighing 28 g While all granules are seen in the freeze dried cryostate section, immersed in liquid paraffin and observed through phase contrast microscope (Fig 2a) approximately only the outer half of the granular ducts are stained with the Bowie stain (Fig 2b), a still narrower zone is stained using iron haematoxylin stain (Fig 2c) (Magnification 30 X)

phology and renin content were studied in submaxillary glands of albino mice, strain Novo, of different ages The results given in Table 1 show that no renin was found in the submaxillary glands of mice weighing from about 6 5 to 11 g (corresponding to an age of about 15 to 22 days) With increasing weight (age), both renin concentration and total amount of renin are increasing, reaching maximal values of 20,000 Goldblatt-Dog Units per g of gland in adult mice

The table also shows that there is a parallel lack of *granules* in the striated ducts in the quite young mice, and that the degree of granulation increases with increasing age The granulation was studied by phase contrast and by light microscopy, using both freeze-dried unstained and fixed, stained preparations In the youngest mice, showing a lesser degree of granulation of the striated ducts (prep 289 in Table 1) nearly all granules were located in the peripheral zone, all of them apparently being stainable In somewhat older mice, granules were found throughout the whole gland Bowie stained granules were almost exclusively seen in the outer half of the gland, and iron haematoxylin stained granules were found in a still narrower peripheral zone (Fig 2a, b, c) In the oldest mice, having the highest renin content, the periphery of the gland was strongly and the central part more lightly stained After microdissection and separation of the peripheral from the central part it was found that extracts of the two fractions had about the same renin content, showing that there is no connection between stainability of the granules and renin content

While a rough quantitation of the granules was possible, a similar quantitation of the amount of *epithelioid cells* in the arterioles of the glands had to be given up because of the paucity of the number of such cells Although in one quite young mouse (274 in Table 1), the glands of which did not contain measurable amounts of renin, a few epithelioid cells were seen, these cells were ordinarily more easily found in grown up animals The few epithelioid cells, which could be found were



Fig 4

The peripheral portion of a freeze dried cryostat section from a submaxillary gland It contains acini intercalated ducts and granulated striated ducts but is devoid of excretory ducts and arterioles (Phase contrast × 100)



Fig 5

Fragment from the

thickness of this preparation might differ somewhat from that of sections used for microdissection In this way (Table 2) it was found that

than in the peripheral areas which are devoid of these cells It is therefore improbable that the epithelioid cells contain renin The table further shows that there is such good agreement between the relation of the relative amounts of 1) granulated ducts and 2) renin concentration in the two fractions, that it is highly probable, that renin is in fact located in the granulated ducts

kidney in being located in vessels containing an elastica interna, which is stained by the Bowie stain

Table 1 also shows that while renin is found in the *kidneys* of young mice at a time, when the submaxillary glands do not contain measurable amounts of renin, the renin content increases in the glands in such a way, that both concentration and total amount of renin in the glands of adult mice greatly exceeds its content in the kidneys, this being in accordance with previous determinations by *Werle et al* (1962)

2 *Relation between Granulated Ducts and Renin in the Submaxillary Glands*

The studies reported in Table 1 speak in favour of a relationship between renin and the granulated striated ducts of the submaxillary glands

TABLE 2

Mouse	Renin concentration in		Amount of granulated tissue as percentage of total tissue		C in percent of P	
	Peripheral fragment (P)	Central fragment (C)	Peripheral fragment (P)	Central fragment (C)	Renin	Granulated area
Pa 1	150	35	33 3	11 4	23	34
Pa 2	91	15	36 0	8 0	17	22
Ra	84	10 7	40 2	4 6	13	11
Sa	112	25	30 0	6 7	22	22

The *left section* shows the renin concentration of peripheral and central fragments of slices, microdissected as shown in Figs 4 and 5 The values are given in percent of the concentration of renin in non microdissected tissue The *middle section* shows the percent amount of granulated tissue in the peripheral and central fragments determined as described under 'Methods" The *right section* shows the relation between the renin concentration and the granulated area in the two fractions the values from the central areas (C) given as percent of the values in the peripheral areas (P) The value of 23 per cent for the amount of renin in the central as compared to the peripheral fragment has been obtained from $\frac{150 \text{ (amount of renin in P)}}{35 \text{ (amount of renin in C)}}$ × 100 The corresponding value of 34 per cent for the content of granulated areas in C as a % of that in P, was obtained from $\frac{11\ 4 \text{ (\% of C as granulated tissue)}}{33\ 3 \text{ (\% of P as granulated tissue)}} \times 100$

In order to obtain more precise information about the renin content of the different structures of these glands, freeze dried sections were microdissected in such a way, that one portion contained only sections of the periphery including acini, intercalated ducts convoluted granular ducts and smaller vessels, devoid of epithelioid cells (Fig 4), while the other portion taken from the same sections contained the excretory ducts with arterioles arteries and veins, with rather small amounts of the other structures (Fig 5) For a rough survey the renin content of sections of non-microdissected tissue was determined, even though the



Fig 4
The peripheral portion of a freeze dried cryostat section from a submaxillary gland It contains acini intercalated ducts and granulated striated ducts but is devoid of excretory ducts and arterioles (Phase-contrast × 100)



Fig 5
Fragment from the central part of a section from a submaxillary gland isolated by microdissection *prepared as that* of Fig 4 The fragment contains a bifurcating excretory duct (d) an arteriole (a) and a venule (v) and includes parts of granulated *ducts some of which* are marked by arrows (Phase contrast × 100)

thickness of this preparation might differ somewhat from that of sections used for microdissection In this way (Table 2) it was found that the concentration of renin was much smaller (from 13 to 23 per cent) in the tissue from the central parts which include the region containing the epithelioid cells than in the peripheral areas which are devoid of these cells It is therefore improbable that the epithelioid cells contain renin The table further shows that there is such good agreement between the relation of the relative amounts of 1) granulated ducts and 2) renin concentration in the two fractions, that it is highly probable, that renin is in fact located in the granulated ducts

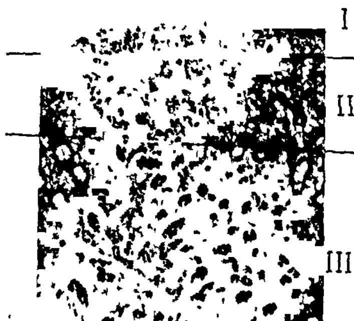


Fig 6

Tangentially cut, freeze dried liquid paraffin treated cryostat section from the sub maxillary gland of a young mouse weighing 16 g By microdissection 3 zones are isolated

I) an outer zone mainly including acini and intercalated ducts,
II) an intermediary zone, including both acini and ducts most of which are agranular striated ducts and
III) an inner zone containing both acini and ducts most of which are granulated (Phase contrast × 100)

TABLE 3

Mouse	Outer zone	Intermediary zone	Inner zone
R + S	5 %	40 %	
T	2 %		61*
Y	≦ 2 %	20 %	100
Aa	4 %	15 %	67

Renin concentration of outer intermediary and inner zone in slices microdissected as shown in Fig 6 The values are given in per cent of the concentration in non microdissected tissue The asterisk marks the renin concentration of the extract of an inner zone containing parts of the intermediary zone

3 *Relation between Morphology and Renin Content in the Outer Zones of the Submaxillary Glands of Young Mice*

In order to obtain further information about the location of renin in the submaxillary glands 10 μ thick slices of the glands of young mice were cut rather tangentially to the surface Such slices (Fig 6) contain 1) an outer zone, mainly including acini and with only very few ducts, 2) and intermediary zone including both acini and ducts, most of which

are intercalated and agranular striated ducts, and 3) an inner zone, containing both acini and ducts, most of which are granular The figure shows how these three zones could be separated by microdissection, and Table 3 gives the results of determinations of the renin concentration of extracts of such preparations While the outer zone contained only few (2 to 5) per cent of the concentration found in extracts of non-microdissected sections, the intermediary zone contained 15 to 40 per cent and the inner zone 67 to 100 per cent These results make it highly probable that both acini and non granulated ducts contain very little—if any—renin, and that renin is located in the granulated ducts

DISCUSSION

All three parts of the present study on the location of renin in the submaxillary glands make it highly probable that renin is located in the granulated ducts, and that the few epithelioid cells contain only little—if any—renin As previous studies on the location of renin in the kidney (*Bing & Kazimierczak* 1964, *Bing* 1964) have rendered it probable that ren [illegible]

[illegible] ctional similarities between the granulated ducts of the salivary glands and the distal tubules in the kidney

SUMMARY

The location of renin (or a renin like substance) in the submaxillary glands of albino mice was studied in three different types of experiments

1) by comparison of morphology and renin content in glands during the post natal development,

2) by comparison of the renin content of peripheral parts, (mainly consisting of acini and granulated ducts, but lacking epithelioid cells and greater excretory ducts and vessels) with the content of isolated central parts of the glands (which mainly contain vessels with epithelioid cells and greater excretory ducts, but only smaller amounts of acini and granulated ducts), and

3) by comparison of the renin content in fragments of the periphery of the gland separated by microdissection, and containing I) mainly acini and II) acini and mostly non granulated d [illegible]

[illegible] ghly probable that renin is formed in the granulated ducts while the acini, the vessels containing epithelioid cells, and the greater excretory ducts seem to contain little—if any—renin

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The State Veterinary Medical Institute Stockholm

A TAXONOMIC STUDY OF THE GENUS *PASTEURELLA* USING A NUMERICAL TECHNIQUE

By

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Received 10 xi 64

The genus *Pasteurella* at present included in Bergey s Manual (*Breed, Murray & Smith* 1957) as a member of the family *Brucellaceae*, is listed as containing nine species, *multocida, septicaemiae, haemolytica, anatipestifer, pestis, pfaffi, pseudotuberculosis, tularensis* and *novicida* Since publication of the current edition of the Manual the last two species on this list have on account of their special cultural requirements been re classified as *Francisella tularensis* (*Olsufiev, Emelyanova & Danayura* 1959, *Philips & Owen* 1961) and F novicida (*Owen et al* 1963), using the generic name originally suggested by *Dorofeev* (1947) Three others, *Past pfaffi*[1], *septicaemiae* and *anatipestifer* are of dubious status the first is almost indistinguishable from *Past pseudotuberculosis* while the latter two, both gelatine liquefiers, are excluded if only by the Manual s own definition of the genus which unequivocally states that gelatine is not liquefied

The grouping of the remainder, *Past multocida, haemolytica, pestis* and *pseudotuberculosis* and of certain more recently described forms, presents further difficulties *Past multocida* (syn *Past septica*) which includes the organisms of bovine haemorrhagic septicaemia a common form of pneumonia in pigs fowl cholera and a variety of other disease conditions in mammals and birds, though biochemically (*Smith* 1958) and serologically (*Roberts* 1947, *Carter* 1955 *Namioka & Murata* 1961) heterogeneous is usually regarded as a single species Its relationship with *Past haemolytica* (*Newsom & Cross* 1932), a group of bacteria associated with some forms of pneumonia in cattle and sheep, is however not clear although there are some resemblances between the two The question of the systematic position and species rank of *Past haemolytica* has been further complicated by the isolation from sheep of two distinct types A and T (*Smith* 1961) and from the human respiratory tract of another group of superficially similar strains

[1] The American Type Culture Collection has no *Past pfaffi* on its lists The National Collection of Type Culture of Great Britain has one culture labelled 1102 isolated by Dr F *Pfaff* from an unstated source it was received in 1921 and is a Past pseudotuberculosis group I

termed *Past haemolytica* var *ureae* (*Henriksen & Jyssum* 1960 1961) *Past pneumotropica*, first obtained from the lungs of mice (*Jawet* 1950) and later from humans (*Henriksen* 1962) is also of uncertain status but resembles in many respects *Past multocida*

The close relationship of the plaque bacillus, *Past pestis*, with *Past pseudotuberculosis*, causal agent of pseudotuberculosis in rodents birds and other animals, has been well authenticated (*Girard* 1953 *Pollitzer* 1954, *Thal* 1956, *Parnas* 1961) but many properties of these two species tend to set them apart from the other members of the genus A further type isolated from various sources including pseudotuberculosis in chinchillas and lymphadenitis mesenterica in man and temporarily named '*Pasteurella X*" (*Daniels & Goudzwaard* 1963) appears to be related to but distinct from *Past pseudotuberculosis* (*Knapp & Thal* 1963 *Mollaret & Chevalier* 1964)

The classification techniques reviewed by *Sneath* (1962) and variously termed numerical, arithmetical computer or Adansonian taxonomy have been widely applied in bacterial systematics and are of particular value in the study of heterogeneous and closely related groups On one occasion a collection of *Past multocida* strains was analysed (*Talbot & Sneath* 1960) but the genus Pasteurella has otherwise been neglected The present communication describes an attempt to elucidate the relationships between *Past multocida, haemolytica, haemolytica* var *ureae, pneumotropica, pseudotuberculosis pestis* and X by the use of a numerical technique

MATERIALS AND METHODS

Cultures

25 strains were used and for the purpose of the study were temporarily labelled a to y details of their sources are given below

Past pseudotuberculosis

a Turkey pseudotuberculosis *F Thal Stockholm* (14/I)
b Hare (30/II)
c Mink (43/III)
d Hare (32/IV)
e Hare (25/V)

Past haemolytica

f — C Sm h E1n rgh 97 pe A)
g ype A)
h pe T)
i T)
j (158 type A)

Past X

k Human mesenteric lymphadenitis *S Winblad* Malm (897)
l *H B ht* Zurich (200)
m *H Becht* Zurich (18
n *sig* Zurich (2533)
o) *Siegmann* Celle (268)

Past multocida

p Buffalo haemorrhagic septicaemia *E Wijewanta* Peradiniya (561)
q Buffalo haemorrhagic septicaemia *G Sharma* Mukteswar (S 138)
r Pig pneumonia *J Smith* London (RVC 518)
s Cat condition unknown *V Stamatin* Bucarest (4118)
t Sheep pneumonia. *L Badiali* Perugia (5)

Past pneumotropica

u Human nose *S Henriksen* Oslo (953/60)
v Human throat *S Henriksen* Oslo (4225/60)
w Human dog bite *J Talbot* London (12/Venn)

Past pestis

x Human plague vaccine strain *G Girard* Paris (EV 76)

Past haemolytica var ureae

y Human ozaena *S Henriksen* Oslo (320 59)

No cultures of *Past pfaffi septicaemiae* or *anatipestifer* were available for study

Tests

All cultures were examined by the foll wing tests the methods employed being those described by *Kauffmann* (1954) *Ewing* (1960) and *Edwards & Ewing* (1962) unless otherwise stated

on litmus milk M R test production of indole acetoin catalase oxidase (using 0 5 per cent aqueous tetramethyl p phen ... chloride) urease gelatinase an ... production of ac d ... cell ... inu ... sor ... Ox ...

Numerical Analysis

The reactions of the strains were scored in columns on strips of paper using scoring method III of *Beers et al* (1962) for each test a + sign was entered to indicate the result any preceding character states being scored—and any subsequent ones O Percentage similarity coefficients (S) were calculated mentally for each possible pair of strains by laying the record strips side by side counting n the number of times + signs coincided and n the number of times + and — signs coincided and substituting in the formula

$$S = \frac{100\,n}{n + n}$$

0 signs were ignored

The strains were s rted manually by ... level ...

termed *Past haemolytica* var *ureae* (*Henriksen & Jyssum* 1960, 1961) *Past pneumotropica*, first obtained from the lungs of mice (*Jawetz* 1950) and later from humans (*Henriksen* 1962) is also of uncertain status but resembles in many respects *Past multocida*

The close relationship of the plague bacillus, *Past pestis*, with *Past pseudotuberculosis*, causal agent of pseudotuberculosis in rodents birds and other animals, has been well authenticated (*Girard* 1953 *Pollitzer* 1954, *Thal* 1956, *Parnas* 1961) but many properties of these two species tend to set them apart from the other members of the genus A further type isolated from various sources including pseudotuberculosis in chinchillas and lymphadenitis mesenterica in man and temporarily named "*Pasteurella X*" (*Daniels & Goudzwaard* 1963) appears to be related to but distinct from *Past pseudotuberculosis* (*Knapp & Thal* 1963 *Mollaret & Chevalier* 1964)

The classification techniques reviewed by *Sneath* (1962) and variously termed numerical, arithmetical, computer or Adansonian taxonomy have been widely applied in bacterial systematics and are of particular value in the study of heterogeneous and closely related groups On one occasion a collection of *Past multocida* strains was analysed (*Talbot & Sneath* 1960) but the genus Pasteurella has otherwise been neglected The present communication describes an attempt to elucidate the relationships between *Past multocida, haemolytica, haemolytica* var *ureae, pneumotropica, pseudotuberculosis, pestis* and "X" by the use of a numerical technique

MATERIALS AND METHODS

Cultures

25 strains were used and for the purpose of the study were temporarily labelled a to y, details of their sources are given below

Past pseudotuberculosis

a	Turkey, pseudotuberculosis *F Thal* Stockholm	(14/I)
b	Hare	(30/II)
c	Mink	(43/III)
d	Hare	(32/IV)
e	Hare	(25/V)

Past haemolytica

f	Sheep pneumonia *G Smith* Edinburgh 97 type A)	
g	Sheep pneumonia *J Smith* London (RVC 1057 type A)	
h	Lamb septicaemia *G Smith* Edinburgh	(1190/1 type T)
i	"	(121 type T)
j	"	(158 type A)

Past X

k	Human mesenteric lymphadenitis *S Winblad* Malmö	(897)
l	[illegible] Zurich (200[illegible]	
m	[illegible] Zurich	(18)
n	[illegible]	(2533)
o	[illegible] Celle	(268)

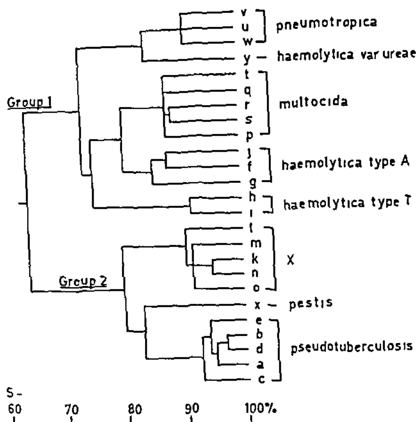


Fig 2
Dendrogram 25 Pasteurella strains

cies linked at 75 to 85 per cent similarity (Fig 2) All strains of a given species appeared in the same cluster in the re-arranged list and, except for those of *Past haemolytica*, were linked by S values ranging from 78 per cent (*multocida*) to as high as 93–97 per cent (*pseudotuberculosis*) In general the Group 2 species were the more homogeneous, as shown in Fig 1 by the denser shading of the triangular areas delineating clusters, and were the more sharply defined Within Group 1, the two strains of *Past haemolytica* type T formed a small, homogeneous sub group (S = 92 per cent) but were linked to type A only at the relatively low level of 76 per cent

Although the principal object of the study was not to examine the differential reactions of the various *Pasteurella* species, a summary of the properties of the strains may be of value and is given in Tables 1 2 and 3

Table 1 shows the results of a variety of biochemical and other tests *Past pseudotuberculosis* and "*Past X*' reacted to these in a similar

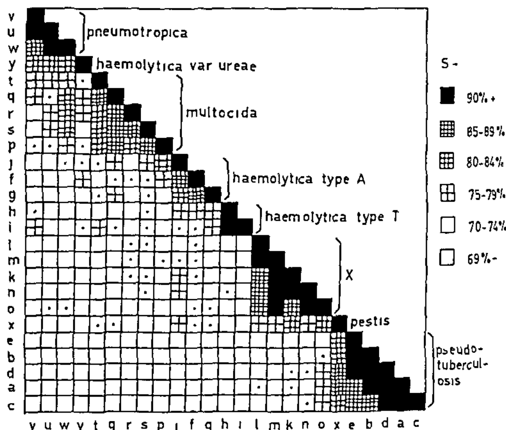


Fig 1
Similarity matrix 25 Pasteurella strains

RESULTS

The calculated values of S ranged from 51 per cent (strains d and i) to 97 per cent (b and d) In accordance with the usual practice in publications of this nature the cumbersome table of similarity values has been omitted but, using these values, the strains were re-arranged into final order v u w y t q r s p j f g h i l m k n o x e b a c Fig 1, based on the rearranged list, is a similarity matrix giving in symbol form approximations of all S values and thus showing cross relationships between strains and clusters of strains Fig 2, a dendrogram, is also based on this list

Two main groups may be discerned, Group 1 containing all strains of *Past multocida, haemolytica, haemolytica* var *ureae* and *pneumotropica*, and Group 2, composed of *Past pseudotuberculosis*, "X" and *pestis* This is best seen in Fig 2 which shows the two groups linking at 65 per cent similarity Fig 1 gives the additional information that there was little cross relationship between the two groups the rectangular area lv, li, ei ev, contains only three entries indicating similarities greater than 75 per cent, and most are less than 70 per cent

Within the two main groups, the clusters representing different spe-

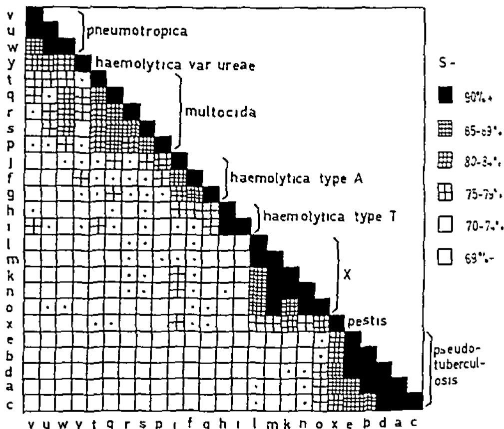


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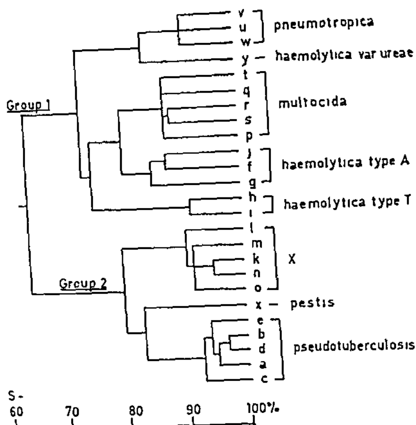


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TABLE 1

Biochemical and other Reactions

Character	Pneumotropica	Haemolytica var ureae	Multocida	Haemolytica type A	Haemolytica type T	[illegible]	Pestis	Pseudotuberculosis
Motility, 22° C	—	—	—	—	—	+	—	+
Haemolysis	—	+	—	+	+	—	—	—
MacConkey, growth	—	—	—	+	+	+		+
Indole	+	+	+	—	—	—	—	—
V P	—	+	—	—	—	—	—	—
M R	—	+	—	—	—	+	+	+
Urea	+	+	—	—	—	+	—	+
KCN	—2	—	+4	—	—	—3	—	—
Oxidase	+	+	+	+	+	—	—	—
Litmus milk	—	Alk	—	—	—	—	Clot	Alk
Lysine decarboxylase	—	—	—	—	—	—	—	—
Argenine decarboxylase	—	—	—	—	—	—	—	+3
Ornithine decarboxylase	—	—	+	—	—	+	—	—
Strains examined	3	1	5	3	2	5	1	5

Catalase all strains + except *Past haemolytica* var *ureae* (trace +), gelatine citrate all strains – Where results varied, the majority reaction is given, followed by the number of strains which reacted thus Alk litmus milk alkaline

TABLE 2

Fermentative Reactions

Substrate	Pneumotropica	Haemolytica var ureae	Multocida	Haemolytica type A	Haemolytica type T	[illegible]	Pestis	Pseudotuberculosis
Aesculin	—	—	—	—	—	—	—	+
Adonitol	—	—	—	—2	—	—	—	+
Amygdalin	—2	—	—	—2	+	—3	—	—
Arabinose	—	—	—	+	—	+	+	+
Cellobiose	—	—	—	—	+	+	—	—
Dextrin	+	+	—4	+2	—	+	—	+
Galactose	+	+	+	+	—	+	+	+
Glycerol	—2	—	+3	+	+	+	—	+
Glycogen	—	—	—	+		—	—	—
Inositol	—	—	—	+2	+1	—	—	—
Maltose	+	+	—4	+	+	+	+	+
Mannitol	—	+	+	+	+	+	+	+
Mannose	+	+	+	+2	—	+	+	+
Raffinose	+2	—	—	—	—	—	—	—
Rhamnose	—		—	—		—	—	+
Salicin	—	—		—	+	—	+	+
Sorbitol	—	+	—3	+	+	+3	—	—
Starch	+	—	—	—2		—	—	+3
Sucrose	+	+	+	+	+	+	—	—
Trehalose	+		+3	—	+	+	+	+
Xylose	—	—	+4	+	—	+	+	+
Strains examined	1	1	5	3	2	5	1	5

Glucose, fructose all strains + melezitose inulin dulcitol lactose all strains — Where results varied the majority reaction is given followed by the number of strains which reacted thus

manner except for the decarboxylase tests and litmus milk *Past multocida* was distinguished from *Past pneumotropica* only by the ornithine decarboxylase and urea tests and the two types of *Past haemolytica* gave identical reactions The oxidase test was uniformly positive with Group 1 strains and negative with Group 2

TABLE 3

Antibiotic Sensitivity Tests

Antibiotic	Pneumotropica	Haemolytica var ureae	Multocida	Haemolytica type A	Haemolytica type T	X	Pestis	Pseudotuberculosis
Bacitracin	$-^{2}$	$\pm$	$\pm^{3}$	$\pm^{2}$	V	$-$	$-$	$-^{4}$
Chlortetracycline	+	+	+	+	+	+	+	+
Erythromycin	+	+	+	+	+	$-^{4}$	$-$	$-^{4}$
Furazolidone	+	+	+	+	+	$+^{3}$	+	+
Neomycin	+	$-$	$+^{3}$	$\pm^{2}$	$\pm$	$\pm^{4}$	+	$+^{4}$
Nitrofurantoin	+	+	+	+	+	+	+	+
Novobiocin	$+^{2}$	$\pm$	$\pm^{4}$	$-$	$-^{1}$	$-$	$-$	$-$
Oleandomycin	$+^{2}$	$-$	$-^{3}$	$-$	$\pm$		$-$	$-$
Oxytetracycline	+	+	+	+	+	+	+	+
Penicillin	+	+	$+^{4}$	+	+		$-$	$\pm^{4}$
Polymyxin B	+	+	$\pm^{4}$	$\pm^{2}$	$\pm$	+	$-$	V
Streptomycin	+	+	+	+	+	+	+	+
Sulphafurazole	$-$	+	$+^{4}$	$+^{2}$	+	$-$	$-$	$-$
Tetracycline	+	+	+	+	+	+	+	+
Strains examined	3	1	5	3	2	5	1	5

Where results varied the majority result is given followed by the number of strains which reacted thus V results too variable for summary
$-$ Inhibition zone 0 5 mm wide or less
$\pm$ Inhibition zone 0 6 to 3 0 mm wide
+ Inhibition zone 3 1 mm wide or more

No comment on the reactions of *Past pestis* and *Past haemolytica* var *ureae* is made since only one strain of each was examined

Fermentation reactions are summarized in Table 2 *Past pseudotuberculosis* and '*Past* X' differed consistently in their action on aesculin adonitol, cellobiose, rhamnose salicin and sucrose and less consistently with sorbitol and starch, while *Past multocida* and *pneumotropica* were distinguishable by their reactions with dextrin, maltose mannitol starch and xylose Cellobiose, galactose, glycogen, salicin and trehalose fermentation tests were among those for which types A and T of *Past haemolytica* gave different results

Certain of the antibiotic sensitivity tests (Table 3) are also of interest *Past pseudotuberculosis* and 'X' for example tended to be erythromycin and novobiocin resistant in contrast to *multocida* and *pneumotropica*, while *Past multocida* and *pneumotropica* showed differences in their sensitivity to sulphafurazole, oleandomycin and bacitracin

DISCUSSION

The major finding of interest was the clear division into Group 1 (oxidase positive) and Group 2 oxidase negative) The low overall similarity between the two groups gives justification for *van Loghem's* (1945, 1946) proposal of a separate *Yersinia*, to accomodate the causal organisms of plague and pseudotuberculosis, and in subsequent discussion these two species will be referred to as *Yersinia pestis* and *Y pseudotuberculosis respectively* *Thal* (1954) considered that *Yersinia* might well be classified within the family *Enterobacteriaceae* and though the present work offers no help on this point it is noteworthy that *Sneath & Cowan* (1958), in a more general computer study of bacteria, placed *Y pestis* between *Escherichia* and *Klebsiella*

The results confirm *Frederiksen's* (1964) assignment of the so-called "*Pasteurella X*" to the same genus, *Yersinia*, they also afford valuable supportive evidence for its species status since the strains examined formed a group whose homogeneity equalled that of *Y pseudotuberculosis* but which was as clearly differentiated from *Y pestis* and *Y pseudotuberculosis* as these two species were from each other (Figs 1 and 2) On grounds of prior description (*Schleifstein & Coleman* 1943) *Frederiksen* favoured the specific epithet *enterocoliticum* for the "X" group but as the two *enterocoliticum* strains available to him were atypical in producing indole, a character of some weight in conventional taxonomy, this question might perhaps be left open for further study, meanwhile "*Yersinia X*" would serve as a convenient label

The residue, Group 1 (oxidase positive), may be regarded as constituting the genus *Pasteurella* Here the differentiation of the sub groups is less sharp than in Group 2 (*Yersinia*), peripheral strains tending to possess intermediate properties and thus causing the sub groups to blend (See Fig 1) *Past multocida* appears as a somewhat heterogeneous cluster of strains all showing about 85 per cent similarity with each other and probably therefore acceptable as a species *Talbot & Sneath* (1960) in a computer study of *Past multocida* found no reason to subdivide the group but their collection of strains was by no means representative and several authors have noted biochemical and serological variations between strains isolated from the different host species (*Roberts* 1947, *Carter* 1955, *Smith* 1958, *Namioka & Murata* 1961) Further work in the form of a detailed comparative study of strains from a wide variety of mammalian and avian hosts is still needed to investigate the validity of the *formae speciales* recognized by earlier authors under such specific epithets as *boviseptica*, *suiseptica*, *aviseptica* and others

The similarity matrix, Fig 1, gives the better indication of the affinities of *Past multocida* within Group 1 and shows its main cross relationships to be with *Past pneumotropica* (The impression given by Fig 2 may be misleading here since the dendrogram depends on similarities

[illegible] clusters only) The single strain of *Past haemo-*

of this bacterium also found its affinities with [illegible] slight and chose to call it *Past ureae*, a decision justified by the present results

The other finding of interest concerns *Past haemolytica* Smith (1961) recognized two types, A (*arabinose positive*) and T (*trehalose positive*) distinguished by colonial, fermentative and other properties In this study, although the two types appeared side by side in the final classification, they differed markedly from each other, linking at as low as 75 per cent similarity (Fig 2) The differences were largely fermentative (Table 2) but a more detailed taxonomic investigation of this group might well furnish grounds for regarding A and T as separate species rather than types of *Past haemolytica*

The study reported, like others of its kind, illustrates the value of the numerical technique in bacterial systematics as an adjunct to more conventional taxonomic procedures Though time-consuming, the method had several valuable features it is objective (at least to the extent that selection of material and characters for examination can be rendered objective) it enables the systematist to utilize, if he wishes, all available data concerning his material rather than a few empirically chosen properties, and the affinities of the microorganisms studied can be expressed in a quantitative form Provided the number of strains is kept reasonably low, much useful work can be accomplished without recourse to calculating devices The maximum number of strains which might conveniently be handled in this way would seem to be about 25, involving calculation of some 300 values for S for larger problems electronic computation is essential Most work in this field has so far been confined to cultural and biochemical characteristics but there is no fundamental reason why serological phage typing and pathogenicity tests should not also be used although there are difficulties in presenting the results of such tests in a form suitable for analysis, their inclusion would greatly increase the usefulness of the technique

SUMMARY

25 cultures originally identified as *Pasteurella* species, were classified by a numerical (Adansonian) method, and fell into two main groups Group 1 (oxidase positive) comprised *Past multocida* and *pneumotropica*, which were related, the two types of *Past haemolytica*, A and T, which differed markedly from each other, and *Past haemolytica* var *ureae* the latter resembled *Past pneumotropica* rather than *Past haemolytica* and the name *Past ureae* seems more appropriate It is suggested that Group 1 should be regarded as constituting the genus *Pasteurella* and that Group 2 (oxidase negative), containing

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Virus Dept B National Institute of Public Health Oslo (Head A Harboe MD)
and Dept of Microbiology Dental Faculty (Head J Jonsen MD)
University of Oslo Norway

INVESTIGATIONS OF SOME EFFECTS OF HUMAN SALIVA ON INFLUENZA VIRUS

2 *Kinetics of Haemagglutination Inhibition A Comparison of Unspecific Inhibition by Saliva and Inhibition by Immune Serum*

By

GUNNAR ROLLA

Received 23 xi 64

The inhibitors isolated from extracts of ovine and bovine sublingual glands are glycoproteins containing sialic acids and amino sugars (2 3) They are inactivated by receptor destroying enzyme (RDE)

Serum immune antibodies against influenza virus are not inactivated by such treatment (8)

The present paper describes some features of the kinetics of the haemagglutination inhibition caused by unspecific inhibitors from human saliva A comparison is made with the kinetics of the haemagglutination inhibition caused by serum immune antibodies

MATERIALS AND METHODS

Virus Influenza virus strains A_2/Japan/305/1957 and B/Lee were employed Virus

venous injections of one ml of high titred allantoic fluid every other day with an interval of one or two weeks between the courses The rabbits were bled for immune serum 7 10 days after the last injection The sera were stored frozen at —20° C.

The serological tests were performed as earlier described (6) The kinetics of the haemagglutination inhibition (HI) process was studied in two ways

1) By allowing saliva to act
paring serial dilutions of
2) In HI tests by incubating
fore the addition of red cell suspension
The titres obtained in the experiments were plotted against time of incubation

EXPERIMENTAL

Equal volumes of saliva and diluted virus containing allantoic fluid were mixed The allantoic fluid had been diluted so that complete in

hibition of virus just took place in the mixture after sufficient time of incubation After incubation at room temperature for different times [illegible] were determined The results are given in [illegible]

[illegible] B Lee compared with [illegible]

TABLE 1

Release of Virus Haemagglutinins from the Virus Inhibitor Complex
HA titres

Time of incubation	Incubation at room temperature with		A_2 Japan + saline	B Lee + saline
	A_2 Japan	B Lee		
To	16	8	256	256
+ 5 minutes	4	1	256	256
+ 10 minutes	<1	<1	256	256
+ 30 minutes	<1	<1	256	256
+ 1 hour	<1	<1	256	256
+ 2 hours	16	4	256	256
+ 4 hours	64	64	256	256
+ 6 hours	256	256	256	256

Procedure To 0 25 ml of saliva was added 0 25 ml of diluted virus containing allantoic fluid Haemagglutinins in the mixtures were estimated after the stated times of incubation

TABLE 2

Change in Inhibitory Activity of Saliva during Incubation with 4 ADS of Virus
HI titres of saliva

Time of incubation	Incubated at room temperature with	
	A Japan	B Lee
To	640	1280
+ 10 minutes	1280	2560
+ 30 minutes	1280	2560
+ 1 hour	1280	2560
+ 2 hours	1280	2560
+ 4 hours	640	640
+ 6 hours	<2	<2

Procedure To 0 25 ml of saliva or saliva dilutions was added 0 25 ml of diluted allantoic fluid containing 4 ADS After incubation for the stated times 0 25 ml of *red cell suspension* was added and HI titre recorded

In Table 2 the kinetics of the haemagglutination inhibition process was followed by HI tests Identical series of diluted allantoic fluid containing 4 ADS of virus and saliva dilutions were prepared After in

creasing time of incubation the red cell suspension was added to the series During the first few minutes of incubation an increasing capacity to inhibit virus was evident After a period of equilibrium the inactivation of the inhibitor took place No activity was present after 6 hours

Similar experiments were then performed with human and rabbit immune sera against A_2/Japan and B/Lee The sera were treated with cholera-filtrate, heated and absorbed with red cells as described (6) In Table 3 the results of the HA-titrations after addition of serum immune antibodies are given No change in activity took place during incubation with virus After incubation for 4 respectively 6 hours the titres remained unchanged

Table 4 shows the results of experiments with HI-technique No change in activity occurred during incubation with virus

TABLE 3

No Release of Virus Haemagglutinin from the Complex of Virus and Immune Serum
HA titres

Time of incubation	Rabbit antiserum +		Human antiserum +		A_2 Japan + saline	B Lee + saline
	A_2 Japan	B Lee	A Japan	B Lee		
HA titres before addition of serum	192	192	192	192	192	192
To	<6	<6	<6	<6	192	192
+ 5 minutes	<6	<6	<6	<6	192	192
+ 10 minutes	<6	<6	<6	<6	192	192
+ 1 hour	<6	<6	<6	<6	192	192
+ 6 hours	<6	<6	<6	<6	192	192

Procedure To 0 25 ml of serum treated with cholera filtrate was added 0 25 ml of diluted virus containing allantoic fluid Haemagglutinins in the mixtures were estimated after the stated times of incubation

TABLE 4

Unchanged Inhibitory Capacity of Human and Rabbit Antisera during Incubation with 4 ADS of A/Japan and B/Lee
HI titres of serum

Time of incubation	Human antiserum +		Rabbit antiserum +	
	A Japan	B Lee	A Japan	B Lee
To	1500	1000	2000	3000
+ 5 minutes	1500	1000	2000	3000
+ 10 minutes	1500	1000	2000	3000
+ 10 minutes	1500	1000	2000	3000
+ 1 hour	1500	1000	2000	3000
+ 4 hours	1500	1000	2000	3000
+ 6 hours	1500	1000	2000	3000

Pro[illegible] treated serum or serum dilutions was added [illegible] taining 4 ADS After incubation for the stated [illegible] as added and HI titre recorded

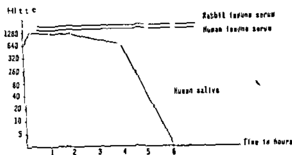


Fig 1

Kinetics of haemagglutination inhibition Rabbit immune serum human immune serum and human saliva incubated with 4 ADS of B/Lee (*Procedure in Tables 2 and 4*)

DISCUSSION

The action of salivary inhibitors on influenza virus was investigated with HA and HI tests The saliva samples had been inactivated by heating at 56° C for 30 minutes Pilot experiments had shown that samples treated in this way had a very stable haemagglutination inhibiting activity The HA- and HI tests were intended to supplement each other, the former giving the amount of free virus particles available after varying times of incubation of the virus saliva mixture (4), the latter giving the smallest volume of saliva necessary for the inhibition of 4 ADS of virus under similar conditions Corresponding experiments were also made with human and rabbit influenza immune sera in order to compare the inhibiting effect of saliva with the inhibition by serum virus antibodies

In Table 1 the kinetics of the inhibition by saliva was examined by HA tests A rapid reduction in free haemagglutinin was observed After 10 minutes all haemagglutinin was inhibited After a period with no free haemagglutinin in the mixture, haemagglutination reappeared after 2 hours reaching full strength after 6 hours of incubation The result shows that two opposite reactions are present in this system The increasing inhibitory capacity during the first few minutes of incubation may be explained in the following way

The haemagglutination inhibition depends on adsorption of virus particles to receptor analogues (1) The maximum adsorption of virus will be reached when a maximum number of collisions between viruses and inhibitor molecules are established This is a time consuming process A study of the interaction between polyoma virus haemagglutinin and a bovine serum inhibitor has been published by *Halperen et al* (3)

The inactivation of the inhibitor as determined by total recovery of the haemagglutinins was completed in 6 hours This inactivation is probably caused by the neuraminidase activity of influenza virus

In Table 2 the experiment was repeated with HI tests At T_0 a saliva dilution of 1 640 was necessary for the inhibition of 4 ADS of A2/Japan

creasing time of incubation the red cell suspension was added to the series. During the first few minutes of incubation an increasing capacity to inhibit virus was evident After a period of equilibrium the inactivation of the inhibitor took place No activity was present after 6 hours

Similar experiments were then performed with human and rabbit immune sera against A_2/Japan and B/Lee The sera were treated with cholera-filtrate, heated and absorbed with red cells as described (6) In Table 3 the results of the HA-titrations after addition of serum immune antibodies are given No change in activity took place during incubation with virus After incubation for 4 respectively 6 hours the titres remained unchanged

Table 4 shows the results of experiments with HI technique No change in activity occurred during incubation with virus

TABLE 3

No Release of Virus Haemagglutinin from the Complex of Virus and Immune Serum
HA titres

Time of incubation	Rabbit antiserum +		Human antiserum +		A Japan + saline	B Lee + saline
	A_2 Japan	B Lee	A Japan	B Lee		
HA titres before addition of serum	192	192	192	192	192	192
T0	<6	<6	<6	<6	192	192
+ 5 minutes	<6	<6	<6	<6	192	192
+ 10 minutes	<6	<6	<6	<6	192	192
+ 1 hour	<6	<6	<6	<6	192	192
+ 6 hours	<6	<6	<6	<6	192	192

Procedure To 0 25 ml of serum treated with cholera filtrate was added 0 25 ml of diluted virus containing allantoic fluid Haemagglutinins in the mixtures were estimated after the stated times of incubation

TABLE 4

Unchanged Inhibitory Capacity of Human and Rabbit Antisera during Incubation with 4 ADS of A_/Japan and B/Lee
HI titres of serum

Time of incubation	Human antiserum +		Rabbit antiserum +	
	A Japan	B Lee	A Japan	B Lee
T0	1500	1000	2000	3000
+ 5 minutes	1500	1000	2000	3000
+ 10 minutes	1500	1000	2000	3000
+ 10 minutes	1500	1000	2000	3000
+ 1 hour	1500	1000	2000	3000
+ 4 hours	1500	1000	2000	3000
+ 6 hours	1500	1000	2000	3000

Procedure To 0 25 ml of cholera filtrate treated serum or serum dilutions was added 0 25 ml of diluted allantoic fluid containing 4 ADS After incubation for the stated times 0 25 ml of red cell suspension was added and HI titre recorded

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STUDIES ON TRANSFORMATION IN MORAXELLA AND ORGANISMS ASSUMED TO BE RELATED TO MORAXELLA

4 *Streptomycin Resistance Transformation between Asaccharolytic* Neisseria *Strains*

By

KJELL BØVRE

Received 26 xi 64

It has been suggested that *Moraxella* and *Neisseria* may be related, because of similar habitat and morphological, cultural and biochemical characteristics (*Henriksen* 1952) The main distinctive trait is the occurrence of rods in *Moraxella* which are often short and coccuslike *Murray & Truant* (1954) however, have pointed out that *Moraxella*, contrary to *Neisseria* divides in only one plane, and suppose that the resemblance is superficial

It has previously been found that *Neisseria catarrhalis* and *Moraxella bovis* have similar DNA base ratios (*Marmur & Doty* 1962) In addition genetic exchange has been observed between *Moraxella* species and *Neisseria catarrhalis* (*Bøvre* 1963) Good reasons therefore exist for the inclusion of *Neisseria catarrhalis* in the studies and the present paper is accordingly centered upon this species As in previous studies (*Bøvre* 1964b 1965) it is aimed at the expression of homogeneity or heterogeneity of many strains in terms of transformation, and these properties will be correlated with conventional criteria to some extent

Neisseria catarrhalis is distinct from all saccharolytic neisseriae hitherto examined both in terms of DNA base composition and in terms of transformation (*Catlin & Cunningham* 1961 *Marmur & Doty* 1962) The omission of the latter group from the studies is therefore considered reasonable

On the other hand the relations between *Neisseria catarrhalis* and other species of asaccharolytic neisseriae in these terms are not known A reservation must be made for *Neisseria flavescens* which seems to belong to the same genetic transfer group as the saccharolytic neisseriae (*Catlin & Cunningham* 1961) An exploration of asaccharolytic neisseriae by genetic means is required for further elucidation of affinities within *Neisseria* Based on this knowledge, transformation experiments will be planned between *Neisseria* and *Moraxella*

10 minutes later only 1/1280 was necessary After incubation for 6 hours a dilution of ½ was unable to inhibit 4 ADS of virus The increasing activity during the first minutes of incubation of virus saliva was thus confirmed by HI-tests

In Tables 3 and 4 the kinetics of the haemagglutination inhibition reaction exhibited by human and rabbit antisera were investigated The sera were treated with cholera-filtrate in order to destroy normal inhibitors No change in the inhibitory capacity could be demonstrated during incubation with virus The progressive part of the inhibitory reaction which was evident in the experiments with saliva could not be found in the virus-antiserum system This indicates that immune antibody reacts faster with virus and that this reaction is irreversible in our test system

A marked difference in the kinetics of the haemagglutination inhibition by immune antisera and that caused by human saliva is thus evident (Fig 1) The difference can be used to investigate the nature of the increased haemagglutination inhibition in saliva which is found after immunization with virus antigen (7)

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microscopical aggregation in the transformation medium The aggregates were studied by means of a dissecting microscope and were found to be very loosely packed and extremely fragile. The usual dilution of the homologous transformation mixture by means of several steps of pipetting was sufficient to break the aggregates almost completely

RESULTS

Description of the Wild Type Strains

All 25 strains consisted of diplococci with adjacent sides flattened and with more than one division plane Some of the strains had smaller cells than the average, but this feature seemed somewhat inconstant in one strain, *Neisseria ovis* 917/60, the occurrence of tetrades was pronounced Giant forms were occasionally seen Rodlike organisms were not observed A common microscopical picture is presented in Fig 1

All strains were Gram-negative, with some resistance to decolorization, similar to that of *Moraxella nonliquefaciens* and *Moraxella bovis* previously described (*Bovre* 1964b, 1965)

The colonies on blood agar after 20 h incubation were generally smaller than those of *Moraxella nonliquefaciens*, 0 5–1 mm in diameter Within these limits, *Neisseria ovis* presented the largest and *Neisseria cinerea* the smallest colonies The colonies were all even, glistening and circular at this stage of growth All strains of *Neisseria catarrhalis* (ex-

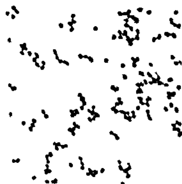


Fig 1
Neisseria catarrhalis Ne 11, stained with Loffler's methylene blue × 960

MATERIAL AND METHODS

15 strains classified as *Neisseria catarrhalis* according to conventional criteria (oxidase positive aerobic asaccharolytic nonhaemolytic nonpigmented Gram negative cocci with more than one division pla
strain Ne 11 received from Dr B W Catlin
of Type Cultures London and strain 8176 f
tion The other 12 strains were isolated from
Rikshospitalet Oslo over a 3 year period (denominator of strain designations tabulated indicates year of isolation)

10 strains of asaccharolytic neisseriae other than *Neisseria catarrhalis* were also included Among these were 2 strains of *Neisseria cinerea* (designated by and received from Dr U Berger) 2 strains of *Neisseria caviae* (strain 10293 from the National Collection of Type Cultures London and strain 14659 (type strain) from the American Type Culture Collection) 3 strains of *Neisseria ovis* (isolated by Dr K Lindqvist from cases of ovine conjunctivitis in 3 different herds over a 6 year period) and 3 strains of *Neisseria flavescens* (strain 8263 from the National Type Culture Collection London and strains 13115 and 13120 (type strain) from the American Type Culture Collection)

sulphide production was applied In the two former media distilled water was replaced by Brain Heart Infusion (Difco) To all three media 10 per cent horse serum was added In the improved medium for nitrite production 0 1 per cent KNO_3 was substituted for the original 0 02 per cent KNO_3 Pigment production was studied on the Loffler slants and on Brain Heart Infusion agar plates during 4 days of incubation All tests were performed at 32 33° C and at 37° C, except for sensitivity tests in which only the

Quantitative streptomy
DNA as described (*Bovr*
with transforming DNA
not exposed to DNA and

Transforming DNA quantities of the order 30–40 µg per ml of the transformation mixture were applied Controls performed with half of this DNA amount did not show deviating transforming effects Values below 5 µg per ml however in some instances gave reduced transformant counts in 15 min DNA exposure

As before mutant and transformant selection was regularly performed at 500 µg streptomycin per ml In selected experiments transformants were also scored at 10 µg streptomycin per ml All kinds of control were not combined in each experiment as the stability of the procedure seemed great 1964b)

rhalis 4103 (40
tinuous DNA ex
tion) Plating w
dition of DNA
formant colonies than did 15 min terminated exposure when the usual phenotype expression period was applied (streptomycin added at the pin point colony stage) With prolongation of the expression period the relative effect of continuous ex
po ason and because DNA saturation
see ons cont nuous DNA exposure can
on to transformation ratios
to quantitative transformation reactions to test the validity of small interstrain transformant counts Thousands of resistant colonies in continuous exposure indicate that a small number of resistant colonies in terminated exposure really represents transformants and not occasional mutants occurring regardless of negative controls In cases where no transformants are detected in the terminated interstrain transformation parallel a negative result also in continuous interstrain DNA exposure indicates that the inter to intrastrain transformation ratio is 10^2–10^3 times lower than the limit obtained (and tabulated)

TABLE 1
Some Characteristics of the Neisseria Strains

Strain	Consistency of colonies	Agglutinability in physiological saline	Growth on the surface of Hugh & Leifson's medium	Growth in citrate	Nitrite production	Urease activity	Haemolysis	Serum liquefaction	Yellow pigmentation
N catarrhalis	Friable	+	+	—	+	—	—	—	—
N catarrhalis	Almost soft	—	V	—	—§	—	—*	—	—
N catarrhalis	Friable	+	+	—	+	—	—	—	—
N catarrhalis 1	Friable	+	+	—	+	—	—	—	—
N catarrhalis 1	Friable	+	+	—	+	—	—	—	—
N catarrhalis 1	Friable	+	+	—	—§	—	—	—	—
N catarrhalis 1	Friable	+	+	—	+	—	—	—	—
N catarrhalis 1	Friable	+	+	—	+	—	—	—	—
N catarrhalis	Friable	+	+	—	+	—	—	—	—
N catarrhalis	Friable	+	+	—	+	—	—	—	—
N catarrhalis	Friable	+	+	—	+	—	—	—	—
N catarrhalis	Friable	+	+	—	+	—	—	—	—
N catarrhalis	Friable	+	+	—	+	—	—	—	—
N catarrhalis	Friable	+	+	—	+	—	—	—	—
N catarrhalis	Friable	+	+	—	+	—	—	—	—
N caviae	Soft	—	+	—	+	—	(+)†	—	—
N caviae	Soft	—	+	—	+	—	(+)†	—	—
N ovis	Almost soft	—	+	—	+	—	+	—	—
N ovis	Friable	+	+	—	+	—	+	—	—
N ovis	Almost soft	—	+	—	+	—	+	—	—
N cinerea	Coherent*	—	—	—	—§	—	—	—	(+)†
N cinerea	Coherent*	+	—	—	—§	—	—	—	(+)†
N flavescens	Coherent*	—	+	—	—§	—	—	—	+
N flavescens	Coherent*	—	+	—	—§	—	—	—	+
N flavescens	Coherent*	—	+	—	—§	—	—	—	+

* Colony consistency like that of micrococci or gonococci
V Irregularly positive and negative
§ Nitrate apparently not attacked as shown by means of Zn powder reduction
* Pronounced greenish discoloration of blood agar on prolonged incubation
† Weakly positive

cept strain 4103), *Neisseria cinerea* and *Neisseria flavescens* had approximately hemispherical colonies, whereas strain 4103 *Neisseria caviae* and *Neisseria ovis* had low, conical colonies The *Neisseria cinerea* colonies were translucent, whereas the remaining strains had colonies varying from almost opaque to semiopaque The opacity was most pronounced centrally, leaving a contrasting clear periphery particularly in the low conical colony type

All strains failed to grow under strictly anaerobic conditions (hydrogen atmosphere) In 0.4 per cent Brain Heart Infusion agar stab culture growth was fairly good down to 5 mm below the surface The strains were all immotile, oxidase positive, and did not produce acid from glucose Other characteristics are listed in Table 1 In some instances it was difficult to observe growth in the original media for indol nitrite and hydrogen sulphide production In the improved media for these purposes, growth was regularly observed The recorded reactions however, were identical in the two sets of media Growth of *Neisseria catarrhalis*, *Neisseria caviae* and *Neisseria ovis* on ascites agar and bovine serum slants was good, whereas the growth of *Neisseria cinerea* and *Neisseria flavescens* on these media was inferior

The strains of *Neisseria catarrhalis* and *Neisseria flavescens* grew as well at 32–33° C as at 37° C, except strain 4103 which seemed to prefer incubation at 37° C *Neisseria ovis* and, in less degree *Neisseria caviae* and *Neisseria cinerea* also preferred the latter growth temperature In no case, however, incubation temperature influenced the results of biochemical and other tests applied The need for a humid atmosphere was not pronounced in any of the strains

The *Neisseria catarrhalis* colonies were considered nonpigmented although they often had a yellowish or brownish tinge *Neisseria caviae* did not reveal pigmentation clearly distinct from the *Neisseria catarrhalis* colony appearance although the Löffler serum cultures of *Neisseria caviae* turned a little more brownish

The strains were all strongly sensitive to penicillin streptomycin chloramphenicol oxytetracycline and erythromycin (Table 2)

Transformation Reactions

Streptomycin sensitive *Neisseria catarrhalis* Ne 11 was applied as recipient in experiments with all 25 strains acting as donors of streptomycin resistance and ratios of inter- to intrastrain transformation were determined The results are listed in Table 3 All *Neisseria catarrhalis* strains except one give high ratios of inter- to intrastrain transformation ranging from 3.0×10^{-1} to 9.4×10^{-1} The deviating strain 4103 reveals ratios from 3.6×10^{-3} to 4.6×10^{-3} in three different experiments with the same recipient strain *Neisseria caviae* and *Neisseria ovis* show a rough uniformity as regards donor activity on the *Neisseria catarrhalis* Ne 11 recipient from 9.8×10^{-6} to 2.0×10^{-5} times that of homologous

mutant DNA These very low interstrain transformant counts were veri fied by high transformant counts in parallel continuous DNA exposure [illegible] from streptomycin sensitive *Neisseria ovis* 199 55 had no [illegible]

There was no activity of the *Neisseria cinerea* and *Neisseria* [illegible] DNA extracts on [illegible] transformation o[illegible] in Table 3 as <2 [illegible] exposure applied to these experiments were also negative This indicates

TABLE 3

Quantitative Streptomycin Resistance Transformation of Neisseria catarrhalis Ne 11 with DNA Extracts from Various Neisseria Strains

Donor strain		Recipient count ml	Interstrain transformants ml	Intrastrain transformants ml	Ratio of inter- to intrastrain transformation
N *catarrhalis*	4103	8 0 10^6	1 4 10^3(137)§	3 6 10^5(358)	3 8 10^{3}
N *catarrhalis*	4103	4.3 10	1 2 10^3(121)	3 4 10^5(34)	3 6 10^{-3}
N *catarrhalis*	4103	5 0 10^6	5 2 10^2(52)	1 1 10^5(112)	4 6 10^{-3}
N *catarrhalis*	8176	3 0 10^8	3 2 10^6(322)	3 8 10^6(383)	8 4 10^{1}
N *catarrhalis*	12910 62	6 0 10^4	1 9 10^3(193)	4 3 10^3(432)	4 5 10^{1}
N *catarrhalis*	13016 62	6 0 10^4	4 0 10^3(398)	4 3 10^3(432)	9 2 10^{1}
N *catarrhalis*	13074,62	6 0 10^4	1 5 10^3(154)	4 3 10^3(432)	3 6 10^{1}
N *catarrhalis*	13074/62	4 1 10^4	6 8 10^2(68)	2 1 10^3(205)	3 3 10^{1}
N *catarrhalis*	13135/62	6 0 10^4	4 1 10^3(408)	4 3 10^3(432)	9 4 10^{-1}
N *catarrhalis*	13430,62	6 0 10^4	2 6 10^3(261)	4 3 10^3(432)	6 0 10^{-1}
N *catarrhalis*	2833/63	6 0 10^4	4 0 10^3(401)	4 3 10^3(432)	9 3 10^{-1}
N *catarrhalis*	2982/63	4 1 10^4	7 9 10^2(79)	2 1 10^3(205)	3 9 10^{1}
N *catarrhalis*	2993 63	4 1 10^4	1 7 10^3(168)	2 1 10^3(205)	8 2 10^{-1}
N *catarrhalis*	7889/63	4 1 10^4	1 8 10^3(181)	2 1 10^3(205)	8 8 10^{1}
N *catarrhalis*	1163 64	4 1 10^4	6 2 10^2(62)	2 1 10^3(205)	3 0 10^{-1}
N *catarrhalis*	1179/64	4 1 10^4	1 0 10^3(101)	2 1 10^3(205)	4 9 10^{-1}
N *catarrhalis*	2424/64	4 1 10^4	1 4 10^3(144)	2 1 10^3(205)	7 0 10^{-1}
N *caviae*	10293	1 8 10^8	6 0 10^1(6)†	3 0 10^6(295)	2 0 10^{5}
N *caviae*	10293	3 5 10^8	7 0 10^1(7)†	5 1 10^6(51)	1 4 10^{5}
N *caviae*	14659	3 5 10^8	7 0 10^1(7)†	5 1 10^6(51	1 4 10^{5}
N *ovis*	199/55	3 5 10^8	5 0 10^1(5)†	5 1 10^6(51)	9 8 10^{-6}
N *ovis*	37 59	3 5 10^8	5 0 10^1(5)†	5 1 10^6(51)	9 8 10^{-6}
N *ovis*	917/60	3 5 10^8	6 0 10^1(6)†	5 1 10^6(51)	1 2 10^{5}
N *cinerea*	165 61	3 5 10^8	$<10^1$(0)*	5 1 10^6(51)	$<$2 0 10^{-6}
N *cinerea*	159 62	3 5 10^8	$<10^1$(0)*	5 1 10^6(51)	$<$2 0 10^{-6}
N *flavescens*	8263	3 5 10^8	$<10^1$(0)*	5 1 10^6(51)	$<$2 0 10^{-6}
N *flavescens*	13115	3 5 10^8	$<10^1$(0)*	5 1 10^6(51)	$<$2 0 10^{-6}
N *flavescens*	13120	1 8 10^8	$<10^1$(0)*	3 0 10^6(295)	$<$3 4 10^{6}

Duration of DNA exposure 15 min Selection concentration 500 µg streptomycin per ml Intrastrain transformants have been scored in simultaneous transformation of the recipient with its own mutant DNA Identical intrastrain transformant counts indicate parallel experiments

§ Figures in brackets indicate means of three plate counts

† In parallel continuous DNA exposure more than 2000 transformant colonies arose on each plate

* In parallel continuous DNA exposure transformants were still not detected

TABLE 2

Antibiotic Sensitivity of the 25 Neisseria Strains
*Ranges of Inhibition Zone Diameters and Approximate Minimum Inhibitory Concentrations (m i c)**

Antibiotic	N. *catarrhalis*		N. *caviae*		N. *ovis*		N. *cinerea*		N. *flavescens*	
	Zones in mm	M i c	Zones in mm	M i c	Zones in mm	M i c	Zones in mm	M i c	Zones in mm	M i c
Penicillin	32–45	0 09 0 002	40 42	0 009 0 005	35 38	0 04 0 02	35–39	0 04 0 01	33–39	0 07 0 01
Streptomycin	23–33	1 0 01	33 34	0 01 0 009	23 25	1 -0 4	28–35	0 1 -0 006	28–37	0 1 -0 003
Chloramphenicol	35 44	0 2 0 02	38–39	0 09 -0 07	33 40	0 3 0 05	33 39	0 3 -0 07	35–45	0 2 -0 02
Oxytetracycline	23 36	0 9 0 01	36 38	0 01 -0 005	24 34	0 6 0 02	31 35	0 06 0 02	27–33	0 2 -0 03
Erythromycin	35 41	0 09 0 02	34	0 1	26 33	1 0 2	25 34	1 -0 1	31–40	0 3 -0 02

* Method of *Ericsson Hogman & Wickman* (1954) M i c values calculated from zone diameters by means of regression equations for each antibiotic (*Ericsson* 1960) M i c given as I U /ml for penicillin as ug/ml for the other antibiotics

reversed reaction presented in Table 3 *Neisseria catarrhalis* 4103 behaves roughly as *Neisseria catarrhalis* Ne 11 as donor of streptomycin resistance to *Neisseria ovis*

... as donors are more active than *Neisseria catarrhalis*

TABLE 5

Quantitative Streptomycin Resistance Transformation of Neisseria ovis 193 55 *with DNA Extracts from Various* Neisseria *Strains*

Donor strain		Recipient count ml	Interstrain transformants ml	Intrastrain transformants ml*	Ratio of inter- to intrastrain transformation
N *ovis*	37/59	3 0 10^7	2 3 10^4(228)§	2 4 10^4(241)	9 5 10^{-1}
N *ovis*	617/60	3 0 10	2 2 10^4(219)	2 4 10^4(241)	9 1 10^{-1}
N *catarrhalis*	Ne 11	5 0 10^8	5 0 10^1(5)†	1 3 10^6(133)	3 8 10^{-5}
N *catarrhalis*	4103	5 0 10^8	4 0 10^1(4)†	1 3 10^6(133)	3 0 10^{-5}
N *caviae*	10293	5 0 10^8	7 3 10^2(73)	1 3 10^6(133)	5 5 10^{-4}
N *caviae*	14659	5 0 10^8	6 8 10^2(68)	1 3 10^6(133)	5 1 10^{-4}
N *cinerea*	165/61	5 0 10^8	$< 10^1$(0)*	1 3 10^6(133)	$<$ 7 5 10^{-6}
N *cinerea*	159/62	5 0 10^8	$< 10^1$(0)*	1 3 10^6(133)	$<$ 7 5 10^{-6}
N *flavescens*	8263	5 0 10^8	$< 10^1$(0)*	1 3 10^6(133)	$<$ 7 5 10^{-6}
N *flavescens*	13115	5 0 10^8	$< 10^1$(0)*	1 3 10^6(133)	$<$ 7 5 10^{-6}
N *flavescens*	13120	5 0 10^8	$< 10^1$(0)*	1 3 10^6(133)	$<$ 7 5 10^{-6}

Duration of DNA exposure 15 min Selection concentration 500 µg streptomycin per ml

on each plate

* In parallel continuous DNA exposure transformants were still not detected

The *Neisseria cinerea* and *Neisseria flavescens* donors, however, do not transform *Neisseria ovis* The ratios $<$7 5 10^{-6} listed and the negative reactions in parallel continuous DNA exposure indicate that eventually fixed ratios of inter- to intrastrain transformation would be of the order 5 10^{-8} or lower

None of the *Neisseria caviae* or *Neisseria cinerea* strains were transformable and experiments with these strains as recipients could therefore not be performed

In *Neisseria flavescens* 13120 and 8263 transformability was too low to permit quantitative transformation, so that continuous DNA exposure had to be used Table 6 presents results of such semiquantitative experiments The reactions observed between the *Neisseria flavescens* strains indicate ratios of inter- to intrastrain transformation of the order 4 10^{-1}, whereas ratios of the order 5 10^{-2} are indicated between *Neisseria flavescens* and *Neisseria cinerea* In an additional experiment (not tabulated) donor DNA from *Neisseria caviae* 14659 had no effect on the recipient *Neisseria flavescens* 13120 (estimated ratio $<$5 10^{-4})

that eventually fixed ratios of inter to intrastrain transformation would be of the order 10 ^{8} or lower

In Table 4 are presented transformation results obtained when using the deviating *Neisseria catarrhalis* strain 4103 as the recipient The transformation ratio between *Neisseria catarrhalis* Ne 11 and 4103 is of the same order as in the reversed reactions shown in Table 3 It is further seen that other members of *Neisseria catarrhalis* share the relatively low order of compatibility with strain 4103 However strain 4103 seems to be a slightly less efficient recipient than donor in transformation reactions with other *Neisseria catarrhalis* strains

TABLE 4

Quantitative Streptomycin Resistance Transformation of Neisseria catarrhalis 4103 *with DNA Extracts from Various* Neisseria *Strains*

Donor strain		Recipient count ml	Interstrain transformants ml	Intrastrain transformants ml	Ratio of inter-to intrastrain transformation
N catarrhalis	Ne 11	5 0 10^{7}	1 4 10^{3}(141)§	5 0 10^{5}(50)	2 8 10^{3}
N catarrhalis	8176	7 5 10^{7}	1 2 10^{3}(120)	7 3 10^{5}(73)	1 6 10^{-3}
N catarrhalis	12910/62	5 0 10^{7}	8 0 10 (80)	5 0 10^{5}(50)	1 6 10^{-3}
N catarrhalis	13016/62	5 0 10	2 8 10"(28)	5 0 10^{5}(50)	5 6 10^{4}
N catarrhalis	13074/62	5 0 10^{7}	6 6 10^{2}(66)	5 0 10^{5}(50)	1 3 10^{3}
N catarrhalis	13135/62	5 0 10	2 7 10^{2}(27)	5 0 10^{5}(50)	5 4 10^{4}
N catarrhalis	13430/62	5 0 10	8 5 10^{2}(85)	5 0 10^{5}(50)	1 7 10^{3}
N caviae	10293	3 5 10	$<$ 10^{1}(0)†	4 0 10^{5}(40)	$<$ 2 5 10^{5}
N ovis	199/55	5 0 10^{7}	$<$ 10^{1}(0)	5 0 10^{5}(50)	$<$ 2 0 10^{5}
N cinerea	159/62	8 0 10^{7}	$<$ 10^{1}(0)	2 0 10^{5}(202)	$<$ 5 0 10^{-5}
N flavescens	13120	3 5 10	$<$ 10^{1}(0)°	4 0 10^{5}(40)	$<$ 2 5 10^{-5}

Duration of DNA exposure 15 min Selection concentration 500 µg streptomycin per ml
Intrastrain transformants have been scored in simultaneous transformation of the recipient with its own mutant DNA Identical intrastrain transformant counts indicate parallel experiments

§ Figures in brackets indicate means of three plate counts

† In parallel continuous DNA exposure approximately 800 transformant colonies arose on each plate

° In parallel continuous DNA exposure transformants were still not detected

Results of transformation attempts between strain 4103 and the other assumed species are also presented in Table 4 There is some indication that strain 4103 may have the same relations as those of *Neisseria catarrhalis* Ne 11 to these species

In Table 5 are presented experiments in which streptomycin sensitive *Neisseria ovis* 199/55 has been exposed to DNAs from other *Neisseria ovis* strains, from *Neisseria catarrhalis* strains representing each of its two entities (in terms of streptomycin resistance transformation) and from all strains of the remaining species of the material The *Neisseria ovis* strains give ratios of inter to intrastrain transformation very close to 1 (9 1 10 ^{1} and 9 5 10 ^{1}) *Neisseria catarrhalis* Ne 11 shows approximately the same low transformation ratio with *Neisseria ovis* as in the

reversed reaction presented in Table 3 *Neisseria catarrhalis* 4103 behaves roughly as *Neisseria catarrhalis* Ne 11 as donor of streptomycin resistance to *Neisseria ovis*

The *Neisseria caviae* donors are more active than *Neisseria catarrhalis* in the transformation of *Neisseria ovis*, the ratios of inter- to intrastrain transformation being 5.1×10^{-4} and 5.5×10^{-4}

TABLE 5

Quantitative Streptomycin Resistance Transformation of Neisseria ovis 199/55 with DNA Extracts from Various Neisseria Strains

Donor strain		Recipient count/ml	Interstrain transformants/ml	Intrastrain transformants/ml	Ratio of inter- to intrastrain transformation
N. ovis	37/59	3.0×10^7	2.3×10^4(228)§	2.4×10^4(241)	9.5×10^{-1}
N. ovis	617/60	3.0×10^7	2.2×10^4(219)	2.4×10^4(241)	9.1×10^{-1}
N. catarrhalis	Ne 11	5.0×10^8	5.0×10^1(5)†	1.3×10^6(133)	3.8×10^{-5}
N. catarrhalis	4103	5.0×10^8	4.0×10^1(4)†	1.3×10^6(133)	3.0×10^{-5}
N. caviae	10293	5.0×10^8	7.3×10^2(73)	1.3×10^6(133)	5.5×10^{-4}
N. caviae	14659	5.0×10^8	6.8×10^2(68)	1.3×10^6(133)	5.1×10^{-4}
N. cinerea	165/61	5.0×10^8	$< 10^1$(0)*	1.3×10^6(133)	$< 7.5 \times 10^{-6}$
N. cinerea	159/62	5.0×10^8	$< 10^1$(0)*	1.3×10^6(133)	$< 7.5 \times 10^{-6}$
N. flavescens	8263	5.0×10^8	$< 10^1$(0)*	1.3×10^6(133)	$< 7.5 \times 10^{-6}$
N. flavescens	13115	5.0×10^8	$< 10^1$(0)*	1.3×10^6(133)	$< 7.5 \times 10^{-6}$
N. flavescens	13120	5.0×10^8	$< 10^1$(0)*	1.3×10^6(133)	$< 7.5 \times 10^{-6}$

Duration of DNA exposure 15 min Selection concentration 500 µg streptomycin per ml Intrastrain transformants have been scored in simultaneous transformation of the recipient with its own mutant DNA Identical intrastrain transformant counts indicate parallel experiments

§ Figures in brackets indicate means of three plate counts

† In parallel continuous DNA exposure more than 2000 transformants colonies arose on each plate

* In parallel continuous DNA exposure transformants were still not detected

The *Neisseria cinerea* and *Neisseria flavescens* donors, however, do not transform *Neisseria ovis* The ratios $<7.5 \times 10^{-6}$ listed and the negative reactions in parallel continuous DNA exposure indicate that eventually fixed ratios of inter- to intrastrain transformation would be of the order 5×10^{-8} or lower

None of the *Neisseria caviae* or *Neisseria cinerea* strains were transformable and experiments with these strains as recipients could therefore not be performed

In *Neisseria flavescens* 13120 and 8263 transformability was too low to permit quantitative transformation so that continuous DNA exposure had to be used Table 6 presents results of such semiquantitative experiments The reactions observed between the *Neisseria flavescens* strains indicate ratios of inter- to intrastrain transformation of the order 4×10^{-3} whereas ratios of the order 5×10^{-6} are indicated between *Neisseria flavescens* and *Neisseria cinerea* In an additional experiment (not tabulated) donor DNA from *Neisseria caviae* 14659 had no effect on the recipient *Neisseria flavescens* 13120 (estimated ratio $<5 \times 10^{-4}$)

TABLE 6

Semiquantitative Streptomycin Resistance Transformation in Neisseria flavescens and Neisseria cinerea

Experiment no	Recipient strain	Donor strain*	Transformant count§
1	*N. flavescens* 13120	*N. flavescens* 13120	148
		N. flavescens 13115	63
		N. flavescens 8263	64
2	*N. flavescens* 13120	*N. flavescens* 13120	405
		N. cinerea 165 61	20
3	*N. flavescens* 8263	*N. flavescens* 8263	395
		N. cinerea 165/61	18
		N. cinerea 159 62	20
4	*N. flavescens* 13120	*N. flavescens* 13120	553
		N. cinerea 165/61	32
		N. cinerea 159 62	29

DNA exposure not terminated before plating Selection concentration 500 µg streptomycin per ml

* 40 µg DNA per ml of the transformation mixture

§ Mean of two plate counts

TABLE 7

The Effect on Transformation Frequency of the Integration of heterologous Streptomycin Resistance Marker in Donor DNA

Recipient strain	Recipient count ml	Donor DNA	Transformants ml
N. catarrhalis 4103	3.0×10^{7}	(4103 Smr)	1.1×10^{4}
		(4103 Smr Ne 11)	9.9×10^{3}
N. catarrhalis Ne 11	6.0×10^{7}	(4103 Smr)	3.2×10^{2}
		(4103 Smr Ne 11)	6.4 × 10[illegible]
N. catarrhalis Ne 11	6.0 × 10	(Ne 11 Smr)	8.6×10^{4}
		(Ne 11 Smr 199/55)	7.9×10^{4}
N. ovis 199/55	5.0×10^{8}	(Ne 11 Smr)	5.0×10^{1}
		(Ne 11 Smr 199/55)	2.7×10^{2}

(4103 Smr) = DNA of streptomycin resistant mutant of *Neisseria catarrhalis* 4103

(4103 Smr Ne 11) = DNA of the same strain after transformation to streptomycin resistance by *Neisseria catarrhalis* Ne 11

(Ne 11 Smr) = DNA of streptomycin resistant mutant of *Neisseria catarrhalis* Ne 11

(Ne 11 Smr 199/55) = DNA of the same strain after transformation to streptomycin resistance by *Neisseria ovis* 199/55

Samples of the donors *Neisseria catarrhalis* Ne 11 and 4103 and all donors of the other species examined, were replicated on plates with 1000 µg streptomycin per ml Without exception all colonies yielded growth at this concentration of streptomycin

The transformant selection concen
gave the same yield of transformant
tion in the usual experiments The reactions ...
eria catarrhalis Ne 11, the donor *Neisseria catarrhalis* 4103 and donors of all the other assumed species were tested in this way So were also the reactions between *Neisseria ovis* and *Neisseria caviae*, and between *Neisseria flavescens* and *Neisseria cinerea*

Extensive replica plating of transformants always showed resistance to 1000 μg streptomycin per ml Transformants were always of the same phenotype as recipients (colony morphology and consistency, haemolysis and nitrate reduction), except for the transferred streptomycin resistance

As in previous heterologous transformation experiments (*Bovre* 1965) ... to ascertain that low ratios of inter- to intra-
strain
loci
between *Neisseria catarrhalis* Ne 11 and *Neisseria ovis* ...
... are collected in Table 7 It is indicated that
...

recipient in a heterologous donor leads to a 2 5 fold increase ... formant count (see *Schaeffer* 1958, *Bovre* 1965)

DISCUSSION AND CONCLUSION

The *Neisseria catarrhalis* strains of this investigation are all in accordance with the vague description given in Bergey's manual (*Branham, Murray & Pelczar* 1957), except for the observed nitrate reduction of most strains which in the manual is considered not occurring in genus *Neisseria* Recent papers however, have shown that this negative criterion is no general rule (*Lindqvist* 1960, *Berger* 1961)

A distinct variant of *Neisseria catarrhalis* has been observed, the nitrate negative and, in terms of conventional criteria, also otherwise deviating strain 4103 This strain deviates in terms of transformation from the other 14 *Neisseria catarrhalis* strains, and the deviation is approximately of the same order as that observed between *Moraxella nonliquefaciens* and *Moraxella bovis* in a previous investigation (*Bovre* 1964a) The remaining *Neisseria catarrhalis* strains express their compatibility with each other in ratios of inter- to intrastrain streptomycin resistance transformation which are consistent with a first degree relationship in these terms (*Bovre* 1964b) One of these strains, 13074 62, is nitrate negative but otherwise typical This strain may have undergone a rather restricted mutational change, with loss of nitrate reducing power

The *Neisseria ovis* strains are few, but in view of their origin, conventional characteristics (*Lindqvist* 1960) and interstrain streptomycin

resistance transformation rates, each strain may be considered representative

Only 2 *Neisseria caviae* strains are included, both type culture collection strains, one of which is the type strain of the species (*Lessel* 1964) The distinct pigmentation of this species described by *Pelczar* (1953) and which is maintained as a diagnostic criterion in Bergey s manual (*Branham, Murray & Pelczar* 1957), was not clearly observed during the present investigation It is believed however, that the strains are representative because of their slight haemolytic activity and also because 2 additional lyophilized strains received from Dr M J Pelczar by Dr K Lindqvist were essentially similar when examined by the author under identical conditions

Neisseria ovis and *Neisseria caviae* have a distinct low compatibility in terms of streptomycin resistance transformation The relationship could unfortunately be examined only in a one way fashion but further indication that they may be related is provided by their similar very low compatibility with *Neisseria catarrhalis*

The 2 strains of *Neisseria cinerea* were examined in early subcultures following reception from the collection of Dr U Berger and are in terms of conventional criteria generally in accordance with descriptions by *Berger & Paepcke* (1962) The greenish discoloration of blood agar observed by these investigators may have connection with different culture conditions e g their use of sheep blood instead of human blood or pH differences Their observations on less penicillin sensitivity of *Neisseria cinerea* than of *Neisseria catarrhalis* are not verified in the two strains

The *Neisseria flavescens* strains are all type culture collection strains The type strain is included (*Lessel* 1964) They are in conventional terms similar to the description given in Bergey s manual (*Branham Murray & Pelczar* 1957) of this species Although only a semiquantitative transformation method could be applied in the study of this species also the genetic results indicate that each strain is representative

One-way transformation reactions between *Neisseria cinerea* and *Neisseria flavescens* show that these two assumed species probably are closely related in terms of streptomycin resistance transformation On the other hand there is a uniform absence of reactivity of *Neisseria cinerea* and *Neisseria flavescens* with the other *Neisseria* strains included in the studies As for the incompatibility of *Neisseria flavescens* with *Neisseria catarrhalis* the observations made by *Catlin & Cunningham* (1961) are verified They place *Neisseria flavescens* in the same genetic transfer group as *Neisseria meningitidis Neisseria flava Neisseria perflava Neisseria subflava* and *Neisseria sicca* In addition *Neisseria cinerea* now seems to be a potential member of this group

Neisseria caviae and *Neisseria ovis* are considered belonging to the same main group as *Neisseria catarrhalis* in terms of streptomycin resistance transformation and will therefore along with *Neisseria*

catarrhalis be offered particular interest in future work on the possible relations between *Moraxella* and *Neisseria*

It is interesting to note that the relations between *Neisseria caviae* *Neisseria ovis* and *Neisseria catarrhalis* in terms of transformation are approximately correspondent to preliminary reported reactions between *Moraxella* strains and *Neisseria catarrhalis* (Bøvre 1963)

SUMMARY

25 asaccharolytic *Neisseria* strains (*Neisseria catarrhalis* 15, *Neisseria caviae* 2, *Neisseria ovis* 3, *Neisseria cinerea* 2 and *Neisseria flavescens* 3), were examined by conventional cultural and biochemical methods and by means of quantitative streptomycin resistance transformation

14 of the *Neisseria catarrhalis* strains behaved in both systems as closely related strains, whereas the remaining strain deviated culturally and biochemically, and revealed a relatively low transformation compatibility with members of the main group (ratios of inter- to intra strain transformation ranging from 5 4 10^{-4} to 3 8 10^{-2})

Neisseria caviae revealed a distinct, but low compatibility with *Neisseria ovis* in these terms (ratios ranging from 5 1 10^{-4} to 5 5 10^{-4})

Very low but distinct transformation reactions were observed between the latter two species and *Neisseria catarrhalis* (ratios ranging from 9 8 10^{-6} to 3 8 10^{-5})

Neisseria cinerea and *Neisseria flavescens* expressed close relationship in terms of streptomycin resistance transformation (semiquantitatively estimated ratios of the order 5 10^{-2}) On the other hand, no compatibility was observed between these two assumed species and *Neisseria catarrhalis*, *Neisseria caviae* or *Neisseria ovis* Because of its compatibility with *Neisseria flavescens* and incompatibility with other asaccharolytic neisseriae, *Neisseria cinerea* may have its proper place in the same genetic transfer group as *Neisseria flavescens*, *Neisseria meningitidis* *Neisseria flava* *Neisseria perflava*, *Neisseria subflava* and *Neisseria sicca*

In terms of streptomycin resistance transformation *Neisseria catarrhalis* *Neisseria caviae* and *Neisseria ovis* are considered belonging to another main group, with possible relation to *Moraxella*

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Branham S E *Murray E G D* & *Pelczar M J* *Neisseria*. Pp 480-485 in Breed R S Murray E G D & Smith N R eds. Bergey s manual of determinative bacteriology 7th ed Baltimore The Williams & Wilkins Co 1957 1094 pp

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Neisseria ovis and *Neisseria caviae* have a distinct, low compatibility in terms of streptomycin resistance transformation The relationship could unfortunately be examined only in a one-way fashion, but further indication that they may be related is provided by their similar, very low compatibility with *Neisseria catarrhalis*

The 2 strains of *Neisseria cinerea* were examined in early subcultures following reception from the collection of Dr U Berger, and are in terms of conventional criteria generally in accordance with descriptions by *Berger & Paepcke* (1962) The greenish discoloration of blood agar observed by these investigators, may have connection with different culture conditions, *e g* their use of sheep blood instead of human blood, or pH differences Their observations on less penicillin sensitivity of *Neisseria cinerea* than of *Neisseria catarrhalis* are not verified in the two strains

The *Neisseria flavescens* strains are all type culture collection strains The type strain is included (*Lessel* 1964) They are in conventional terms similar to the description given in Bergey's manual (*Branham, Murray & Pelczar* 1957) of this species Although only a semiquantitative transformation method could be applied in the study of this species, also the genetic results indicate that each strain is representative

One-way transformation reactions between *Neisseria cinerea* and *Neisseria flavescens* show that these two assumed species probably are closely related in terms of streptomycin resistance transformation On the other hand, there is a uniform absence of reactivity of *Neisseria cinerea* and *Neisseria flavescens* with the other *Neisseria* strains included in the studies As for the incompatibility of *Neisseria flavescens* with *Neisseria catarrhalis*, the observations made by *Catlin & Cunningham* (1961) are verified They place *Neisseria flavescens* in the same genetic transfer group as *Neisseria meningitidis*, *Neisseria flava*, *Neisseria perflava*, *Neisseria subflava* and *Neisseria sicca* In addition, *Neisseria cinerea* now seems to be a potential member of this group

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THE DEMONSTRATION OF *N. GONORRHOEAE* WITH THE AID OF FLUORESCENT ANTIBODIES

3 *Studies by Immunofluorescence and Double Diffusion-in Gel Technique on the Antigenic Relationship between Strains of* N gonorrhoeae

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Ever since the work of *Bruckner & Christeanu* (3) and *Bruck* (4) in 1906, and *Vannod* (41) in 1907 the antigens of *Neisseria gonorrhoeae* have been subjected to a great number of serological investigations The earlier results obtained by agglutination tests have been reviewed by *Wilson & Miles* (42), who conclude that there are probably two main serological types of *N gonorrhoeae* with many intermediate forms In 1954 *Wilson* (43), using agglutination and agglutinin absorption tests, demonstrated eight antigens, four of which behaved as group antigens and four as type specific antigens *Reyn* (31, 32, 33) using complement fixation tests demonstrated a thermostable common gonococcus antigen and she also identified several "type specific" and 'strain specific" partial antigens *Chanarin* (6) demonstrated two antigens in smooth phase gonococcal strains, the first could be absorbed on to red cells and sensitized these cells to the action of a gonococcal antiserum, the second antigen appeared to be primarily responsible for complement fixation *Torrey* (38) regarded the protein fraction of *N gonorrhoeae* as the type specific antigenic constituent but *Casper* (5) and *Uroma* (39) considered that the carbohydrate fraction of the organisms is type specific

It will be seen from the serological studies related above that different strains among *N gonorrhoeae* have antigens in common and that they also possess type specific antigens These investigations have, however not led to a standard serological classification of the gonococci into groups or types in analogy with that of streptococci, pneumococci, salmonellae, or *Escherichia coli* Nor have they led to any practical application, with the *exception of the complement-fixation test* in the so-called gonoreaction The latter reaction has, however, been con-

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was also cultured on two other media which are recommended by the Scandinavian committee on culture and sensitivity determination of gonococci (26) Both these media were made up of 1 1 per cent Bacto agar, human placenta broth with 1 per ... 2 (D F a) 0 3 per cent NaCl and 0 2 per cent Na_2HPO_4 ... ate ... agar ... the

... The agar plates were incubated at 36–37° C in a mois... ghly 8-10 per cent CO_2 In order to be classed as gonococci the organisms should be Gram negative diplococci give a positive oxidase reaction and form acid from dextrose but not from maltose and laevulose They should fail to grow on plain nutrient agar at 22° C

C *Preparation of Gonococcus Antiserum*

1 *Preparation of Antigen*

The strain GC1 isolated from a male with urethritis, was used in the present investigation for the preparation of antigen for immunization of rabbits At the primary isolation in 1962 the strain was lyophilized in a sufficient number of ampoules and these were later thawed and used for culture when necessary If not otherwise stated this strain was not subcultured more than five times before a new ampoule was thawed

The strain was cultured on Difco GC medium (see above) for 16 20 hours in about 8 10 per cent CO_2 at 36–37° C The organisms were then harvested in Seitz filtered 0 01 M phosphate buffered physiological saline of pH 7 2 (PBS) kept in an ice water bath The suspensions were washed once or twice in 20–30 volumes of cold PBS and centrifuged each time at 3 500 r p m for 20 minutes and then suspended in PBS to a concentration corresponding to tubes 7–8 of a *McFarland's* scale (23) The suspensions were pooled and divided into four portions The first, second and third portions were treated as previously described (10), with formalin or heat at 100° C (boiling in a water bath) and 120° C (autoclaving) The organisms in the fourth portion were disintegrated by an MSE 20 Kc ultrasonic oscillator operated at ... was per ... 0 sec The ... is shown ... to as us) ... was centrifuged at 10 000 r p m for 20 minutes and the supernatant (referred to as *sup*) was collected The sediment (referred to as *sed*) was then washed twice in PBS and finally resuspended in PBS to the same volume as before centrifugation The purity of the preparations was tested as described previously (10)

2 *Immunization*

Young albino rabbits weighing 2 2 5 kg were used They were injected with gonococci treated with formalin (f) heat (100° C or 120° C) or ultrasound (us) or with the supernatant (sup) or the sediment (sed) of ultrasonic treated gonococci The rabbits are represented in this paper by 6 animals designated as R f R 100° R 120° R us R sup and R sed The first immunization dose was given together with Freund's adjuvant complete (17) (Difco) as described previously (10) and intravenous injections were started about four weeks later according to an immunization schedule used in an earlier work (10) The rabbits were bled 7 8 days after the last injection and bleedings were also performed before the immunization period

Untreated gonococci were not used for immunization because of their tendency to clot after a few days storage

D *Immunological Analyses*

1 *Fluorescent Antibody Procedures*

The preparation of the globulin fractions of rabbit pre immune and immune sera and conjugation with FITC (lot no 1041 obtained from Sylvana Chemical Corp) (22 36) were carried out as described previously (10)

Smears for fluorescent antibody staining were prepared from cultures of the GC strains included above which had grown for 16–20 hours on Difco GC medium

sidered to be of limited value in the diagnosis of gonorrhoea due to poor specificity and sensitivity

A new approach to the diagnosis of gonorrhoea by serological means was made by *Deacon, Peacock, Freeman & Harris* in 1959 (12) and by *Deacon, Peacock, Freeman, Harris & Bunch* in 1960 (13) They showed that the fluorescent antibody (FA) method (7, 8, 9, 25) could be used for the identification of *N gonorrhoeae* in specimens from both males and females which was confirmed by others (2, 11, 18, 21, 24) In the original work by *Deacon et al* (12) and later by *Deacon* in 1961 (14) it was considered that the antigen responsible for the reaction with fluorescein isothiocyanate (FITC) conjugated antigonococcus globulin was a labile surface antigen, named K-antigen, which was preserved by formalin treatment and destroyed by heat at 120° C This argument was, however, questioned by *Ovčinnikov* (29) In a previous work (10), minor variations in the staining reactions were noted No indication of qualitative or quantitative differences in the antigenic composition could be secured, however, since the antigonococcal sera used in that study were obtained by immunization with antigens pooled from 7-8 gonococcal strains selected at random

In the present work the immunofluorescence and the double diffusion-in gel techniques were compared to obtain information on the cellular location of antigens taking part in fluorescent antibody staining and gel precipitation reactions The influence of heat, different media, prolonged culturing, and subculturing, and the antigenic relationship between gonococcal strains were also studied

MATERIAL AND METHODS

A N gonorrhoeae *Strains*

were selected for investigation
aboratory of the Bacteriological
De cimens obtained from 13 males
an om the National Bacteriological
Laboratory (SBL) in Stockholm The latter strains are used by different laboratories in Scandinavia as reference strains for antibiotic sensitivity determinations of N *gonorrhoeae* and they are included in a work by *Reyn Bentzon & Ericsson* (35) in a comparative investigation of the sensitivity of N *gonorrhoeae* strains to penicillin The strains GC III V and VII in their work correspond to the strains GC19 20 and 21 respectively in the present study

All the strains investigated were tested for antibiotic sensitivity according to the disc method (16) They were sensitive to tetracycline and chloramphenicol (IC 50 = 50 per cent inhibitory concentration ≤ 7 0 µg/ml of tetracycline < 4 µg ml of chloramphenicol) The strains GC2 GC6 GC10 GC14 GC15 GC16 and GC20 showed a decreased sensitivity to penicillin (IC 50 usually 0 7–0 1 IU) and were resistant to more than 400 µg/ml of streptomycin The other strains were sensitive to ≤ 0 05 IU of penicillin and ≤ 3 µg/ml of streptomycin

The strains investigated were preserved and stored by lyophilization

B *Culture of Gonococci*

Difco GC Agar Base enriched with Bacto haemoglobin and Supplement B (15) (subsequently abbreviated Difco GC medium) was used as a standard medium in this investigation In a few experiments the strain GC 1 used as a reference strain

was also cultured on two other media which are recommended by the Scandinavian committee on culture and sensitivity determination of gonococci (26) Both these media were made up of 1 1 per cent Bacto agar, human placenta broth with 1 per cent Na_2HPO_4 ... no 3 (Difco), 0 3 per cent NaCl and 0 2 per cent ... fluid (chocolate ... agar ... the ... ghly

agar" at 22° C.

C *Preparation of Gonococcus Antiserum*

1 *Preparation of Antigen*

The strain GC1 isolated from a male with urethritis was used in the present investigation for the preparation of antigen for immunization of rabbits At the primary isolation in 1962 the strain was lyophilized in a sufficient number of ampoules and these were later thawed and used for culture when necessary If not otherwise stated *this strain was not* subcultured more than five times before a

... once or twice in 20–40 volumes of ...

... oscillator operated at ... nic treatment was per... after 120–150 sec The ... atment, which is shown

2 *Immunization*

Young albino rabbits weighing 2 2 5 kg were used They were injected with gonococci treated with formalin (f) heat (100° C or 120° C) or ultrasound (us) or with *the supernatant* (sup) or the sediment (sed) of ultrasonic treated gonococci The rabbits are represented in this paper by 6 animals designated as R f R 100° R 120° R us R sup and R sed The first immunization dose was given together with Freund's adjuvant *complete* (17) *(Difco)* as described previously (10) and intravenous injections were started about four weeks later according to an immunization schedule used in an earlier work (10) The rabbits were bled 7 8 days *after the last injection and bleedings were also performed before the* immunization period

Untreated gonococci were not used for immunization because of their tendency to ... after a few days storage

D *Immunological Analyses*

1 *Fluorescent Antibody Procedures*

The preparation of the globulin *fractions of* rabbit pre immune and immune sera and conjugation with FITC (lot no 1041 obtained from Sylvania Chemical Corp) (22 36) were carried out as described previously (10)

Smears for fluorescent antibody staining were prepared from cultures of the GC strains included above which had *grown for 18–20 hours* on *Difco GC* medium

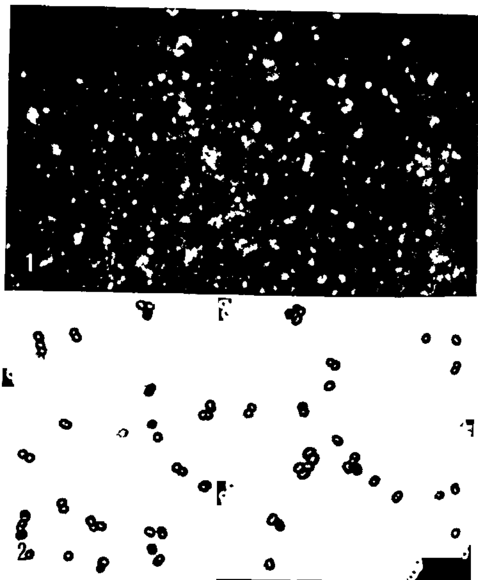


Fig 1 Gonococci (strain GC1) treated with ultrasound for 180 sec and stained with the reference conjugate The morphology of the organisms lost Antigenic material stained (cf Fig 2)

Fig 2 Gonococci of the reference strain (GC1) cultured for 18 hours and stained with the reference conjugate The reaction assessed as 3–4+

Growth from the plates was suspended in PBS to give a turbid suspension corresponding to tubes 2 4 of a McFarland s scale and loopfuls of the suspensions were smeared onto a sufficient number of slides Smears were also prepared from cultures of GC1, which had been treated with heat (40° C–120° C) from cultures which had been subcultured 4, 27 or 102 times, and from cultures which had grown for 12, 30, 40, and 72 hours Finally smears were prepared from GC1 grown for 16–20 hours on chocolate-ascitic agar medium or chocolate horse-serum agar medium

The smears were gently fixed by heat and stai
conjugated globulin solutions which were diluted
initial dilution of 1 2 or 1 4 Washing and mour
microscopy, assessment of the degree of fluorescence
as described previously (10) The FA titres were given as the reciprocal of the highest dilution giving at least a 3 reaction

Antisera The antigonococcal sera obtained from the rabbits ... used undiluted in the experiments The antiserum from rabbit R f (anti GC1 f) was used as a *reference serum* In a few experiments anti GC1 f was used diluted (1 2 1 256)

The *Ouchterlony* technique (27 28) in the

layer were 2 mm wide The
congealed and as a rule doubl
of 4 or 5 mm betwee
The plates were inc
refill ng was done
were recorded or ph
and then dried and stained with amido black in the manner recommended by *Wadsworth* (40)

3 *Agglutination and Complement Fixation Tests*

The agglutination tests were performed as described previously (10) starting with a *serum dilution of 1 2 and using GC1 treated with formalin or by heat at 100° C* or 120° C as antigens The titres are given as the reciprocal of the highest dilution

haemolysis

F *Absorption Experiments*

1 *Absorption with the Reference Stra n (GC1)*

The strain was cultured on Difco GC medium for 16 20 hours Treatment of the organisms with f rmalin and heat and ultrasonic disintegration was carried out as descr bed al ove 0 1 ml of serum or conjugate was mixed with 75–100 mg[1] of the rganisms treated with formalin or heat at 100° C or 120° C After 4–5 hours at +3 4 C the mixture was centrifuged once or twice and the absorbed serum or con jugate was collected Absorption with disintegrated organisms was carried out in

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ment r with the concentrated supernatant alone After 4 5 hours at +3 4° C, cen

[1] Wet weight estimated after centrifugation at 3 500 r p m for 20 min



Fig 1 Gonococci (strain GC1) treated with ultrasound for 180 sec and stained with the reference conjugate The morphology of the organisms lost Antigenic material stained (cf Fig 2)

Fig 2 Gonococci of the reference strain (GC1) cultured for 18 hours and stained with the reference conjugate The reaction assessed as 3 4+

Growth from the plates was suspended in PBS to give a turbid suspension corresponding to tubes 2 4 of a McFarland's scale and loopfuls of the suspensions were smeared onto a sufficient number of slides Smears were also prepared from cultures of GC1 which had been treated with heat (40° C–120° C) from cultures which had been subcultured 4 27 or 102 times and from cultures which had grown for 12 30 40 and 72 hours Finally smears were prepared from GC1 grown for 16 20 hours on chocolate ascitic agar medium or chocolate horse serum agar medium

The smears were gently fixed by heat and stained for 45 60 minutes with the conjugated globulin solutions which were diluted twofold in series in PBS with an initial dilution of 1 2 or 1 4 Washing and mounting of the smears fluorescence microscopy, assessment of the degree of fluorescence and photographs were the same as described previously (10) The FA titres were given as the reciprocal of the highest dilution giving at least a 1 reaction

2 *Agar Gel Diffusion Tests*

ascitic agar medium and chocolate horse serum agar medium (26) New antigen preparations were made every third or fourth week

Antisera The antigonococcal sera obtained from the rabbits mentioned in C2 were used undiluted in the experiments The antiserum from rabbit R f (anti GC1 f) was used as a reference serum In a few experiments anti GC1 f was used diluted (1 2 1 256)

and then dried and stained with amido black in the manner recommended by Wadsworth (40)

3 *Agglutination and Complement Fixation Tests*

The agglutination tests were performed as described previously (10) starting with ...ormalin or by heat at 100° C ...procal of the highest dilution

the same way as before (10) ...as antigen prepared according ... the highest dilution with no haemolysis

E *Absorption Experiments*

Gel diffusion tests and FA tests were carried out after the reference serum (abbreviated *anti GC1 f*) and its FITC-conjugated globulin (abbreviated *FG GC1 f*) had been absorbed in the following way

1 *Absorption with the Reference Strain (GC1)*

The strain was cultured on Difco GC medium for 16 20 hours Treatment of the organisms with formalin and heat and ultrasonic disintegration was carried out as described above 0 1 ml of serum or conjugate was mixed with 75–100 mg[1] of the organisms treated with formalin or heat at 100° C or 120° C. After 4–5 hours at +3-4° C the mixture was centrifuged once or twice and the absorbed serum or conjugate was collected Absorption with disintegrated organisms was carried out in the following way 0 1 ml of serum or conjugate was mixed with the sediment (ob... s) from 75–100 mg[1] of the ...pernatant which had been ...n filter Gottingen) Similar ... mixed either with the sedi...ment or with the concentrated supernatant alone After 4 5 hours at +3 4° C, cen

[1] Wet weight estimated after centrifugation at 3 500 r p m for 20 min

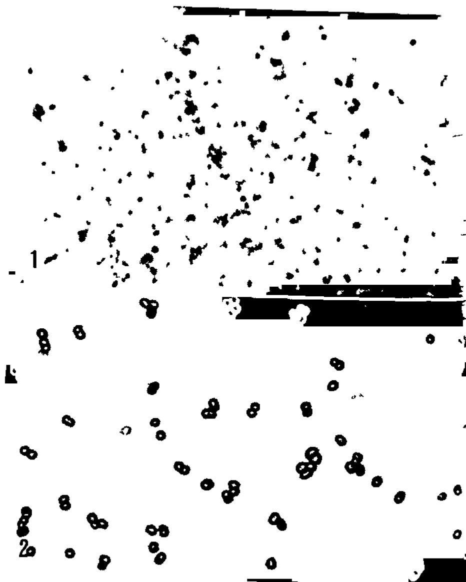


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1 Agar Gel Diffusion Tests

(1 2 1 256) … in a few experiments a

… and then dried and stained with amido black in the manner recommended by Wadsworth (40)

3 Agglutination and Complement Fixation Tests

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fore (10)
according

… (34) The titres are given as the reciprocal of the highest dilution with no haemolysis

1 Absorption with the Reference Strain (GC1)

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[1] Wet weight estimated after centrifugation at 3 500 r p m for 20 min

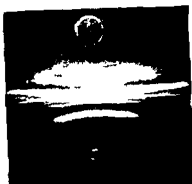


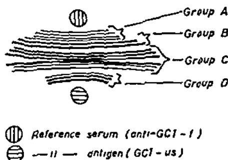
Fig. 1. Gonococci (strain GC1) treated with ultrasound for 180 sec and stained with the reference conjugate. The morphology of the organisms lost. Antigenic material stained (cf. Fig. 2).

Fig. 2. Gonococci of the reference strain (GC1) cultured for 18 hours and stained with the reference conjugate. The reaction assessed as 3–4+.

Growth from the plates was suspended in PBS to give a turbid suspension corresponding to tubes 2–4 of a McFarland's scale and loopfuls of the suspensions were smeared onto a sufficient number of slides. Smears were also prepared from cultures of GC1 which had been treated with heat (40° C–120° C), from cultures which had been subcultured 4, 27, or 102 times and from cultures which had grown for 12, 30, 40, and 72 hours. Finally, smears were prepared from GC1 grown for 16–20 hours on chocolate ascitic agar medium or chocolate horse serum agar medium.

The smears were gently fixed by heat and stained for 45–60 minutes with the conjugated globulin solutions which were diluted twofold in series in PBS with an initial dilution of 1:2 or 1:4. Washing and mounting of the smears, fluorescence microscopy, assessment of the degree of fluorescence and photography were the same as described previously (10). The FA titres were given as the reciprocal of the highest dilution giving at least a 3+ reaction.





A B

Fig 3

Photograph (A) and drawing (B) of the precipitation pattern formed by the reference system Upper basin reference serum (anti GC1 f obtained from rabbit R f) Lower basin reference antigen treated with ultrasonic (GC1 us)

rabbits R 100° and R sed The serum from R sup formed however more precipitation lines with GC1 us than the sera from R sed R 100° and R 120° No precipitation lines were formed when the antisera were tested against Difco GC Agar Base Bacto haemoglobin or Supplement B or against a concentrated extract in PBS of the complete GC medium

3 *Reference System*

For investigations of the reference strain GC1 with the FA method and the gel diffusion technique the antiserum from rabbit R f (anti GC1 f) was found suitable to use as a reference serum because of its high immunological activity

4 *Characterization of the Reference System*

As shown in Table 1 the FITC-conjugated globulin of anti GC1 f (FG GC1 f) gave 3 4+ reactions (Fig 2) with GC1 at a maximal dilution of 1 256 (designated FA titre)

In gel diffusion tests the undiluted anti GC1 f formed at least 15 precipitation lines with the ultrasonic prepared gonococci of GC1 (GC1 us) (Fig 3) Some of the lines in this precipitation pattern were difficult to interpret and the subsequent experiments showed that it was convenient to divide them into four groups referred to as A B C and D (Fig 3) Starting from the serum basin the first four precipitation lines were referred to group A and the next two to group B Six precipitation lines that were formed in the middle between the antiserum and antigen basins were referred to group C and three lines near the antigen basin to group D

Anti GC1 us gave on the whole the same precipitation pattern as anti GC1 f while anti GC1 sup lacked one line in group D and anti

trifugation was carried out and the absorbed serum or conjugate was collected. After these absorptions the serum or the conjugate was diluted 1 2 at the most

Absorption was also performed with formalin treated or ultrasonic treated organisms of the reference strain cultured for 40–72 hours on Difco GC medium or cultured for 16–20 hours on chocolate ascitic agar medium or chocolate horse serum agar medium (26)

2 Absorption with the Strains GC2–21

The strains were cultured on Difco GC medium for 16–20 hours, and absorptions of the reference serum and its FITC conjugated globulin were carried out as described above with organisms treated with formalin or disintegrated with ultrasound

3 Inhibition Tests

One step inhibition tests were performed according to *Redys et al* (30) Undiluted anti GC1 f (uabsorbed or absorbed) was mixed with an equal part of twofold diluted FG GC1 f

RESULTS

1 *Pre-Immune Sera*

Agglutination tests with the pre-immune sera were negative A low complement fixing activity was found in the pre-immune serum from rabbit R-100° (about 30 per cent haemolysis at the dilution of 1 2) but the others were negative The FITC-conjugated pre-immune globulins gave no reactions when they were tested against GC1 In gel diffusion tests a weak precipitation line was obtained with the pre-immune serum from rabbit R-us, but no lines appeared with the others

TABLE 1

The antigonococcal sera Determined by Gel Diffusion Tests and the hod

Designations of rabbits	Treatment of antigen used for immunization	Immunological activity estimated by					
		Agglutination			Compl fix	Gel diff	FA method
		GC1 f*	GC1 100°	GC1 120°	Max inhib	Prec lines formed	3-4 + reactions
R f	Formalin (f)	256	128	128	512	15	256
R us	Ultrasound (us)	256	128	128	1024	15	256
R sup	Supern from us	32	32	32	64	14	64
R sed	Sedim from us	256	128	64	64	10	256
R 100°	Heat 100° C	128	128	64	256	7	128
R 120°	Heat 120° C	64	64	64	256	5	64

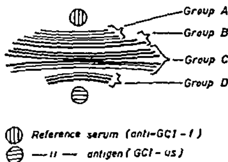
* Treatment of antigens used for agglutination

2 *Antigonococcal Sera*

Table 1 shows the immunological activity of the antigonococcus sera obtained from rabbits immunized with organisms of GC1 treated in different ways The sera from rabbits R-f and R us had the highest immunological activity all through The sera from the rabbits R-120° and R-sup had, on the whole a lower activity than those from the



A



B

Fig 3

Photograph (A) and drawing (B) of the precipitation pattern formed by the reference system. Upper basin reference serum (anti GC1 f obtained from rabbit R f) Lower basin reference antigen treated with ultrasonic (GC1 us)

rabbits R-100° and R-sed The serum from R sup formed, however, more precipitation lines with GC1-us than the sera from R-sed, R-100° and R 120° No precipitation lines were formed when the antisera were tested against Difco GC Agar Base, Bacto haemoglobin or Supplement B, or against a concentrated extract in PBS of the complete GC medium

3 *Reference System*

For investigations of the reference strain GC1 with the FA method and the gel diffusion technique the antiserum from rabbit R-f (anti-GC1 f) was found suitable to use as a reference serum because of its high immunological activity

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In gel diffusion tests the undiluted anti-GC1 f formed at least 15 precipitation lines with the ultrasonic prepared gonococci of GC1 (GC1-us) (Fig 3) Some of the lines in this precipitation pattern were difficult to interpret and the subsequent experiments showed that it was convenient to divide them into four groups, referred to as A, B, C and D (Fig 3) Starting from the serum basin, the first four precipitation lines were referred to group A and the next two to group B Six precipitation lines that were formed in the middle between the antiserum and antigen basins, were referred to group C, and three lines near the antigen basin to group D

Anti GC1-us gave, on the whole, the same precipitation pattern as anti GC1 f while anti GC1-sup lacked one line in group D, and anti-

GC1 sed some lines in group A B and C Anti GC1 100° and anti GC1 120° had a poor precipitating activity and gave some precipitation lines within groups A, C and D

Based on these findings anti GC1 f was chosen as a reference serum because of its complete set of precipitation lines and high FA titre Anti GC1 us was less suitable as its pre immune serum showed un specific serological activity

Three series of *dilution experiments were performed* and the in formation obtained from them is summarized below

In the first series of experiments the antiserum was diluted (1 2 1 256) and the antigen kept constant At 1 2 the precipitation pattern was not influenced except that the line nearest the antiserum basin precipitated very weakly Three of the lines in group A and one in group C were not formed at 1 4 and compared to the reference system *the other lines were located nearer the antiserum basin* At 1 8 the lines in group B and D and five in group C remained and at 1 16 four in group C and two in group D At 1 32 and 1 64 a few diffuse and weak lines belonging to group C and D were formed No lines were formed at the dilution of 1 128

In the second series of experiments the antigen was diluted 1 2 1 256 and the antiserum kept constant At 1 2 one of the lines in group D *was not formed* and at 1 4 two of the lines in group A At 1 8 two lines in group A one in group C and two in group D were not formed and at 1 16 three in group A three in group C and two in group D At 1 32 one line in group B and one in group C remained and at 1 64 only one in group C No lines appeared at the dilution of 1 128

In the third series of experiments serial dilutions (1 2 1 256) of the antigen and antiserum were performed When the reactants were diluted 1 2 the line nearest the antiserum basin was very *faint* At 1 4 the lines in group A and B and one line in group D were not demon strable At 1 8 four lines in group C were formed and at 1 16 1 32 and 1 64 only two No precipitation lines were formed at 1 128

5 *Influence of Heat on the Reference Strain in FA Tests and Gel Diffusion Tests*

Table 2 shows the results of FA staining of GC1 heated at different temperatures with FG GC1 f The FA titres and the morphology of the organisms were compared to those obtained with untreated or formalin treated organisms

Heating to 60° C had no appreciable influence on the FA titre or the morphology After heating to 100° C the gonococci appeared smaller and occurred *more often as single cocci Heating to* 120° C enhanced these effects and lowered the FA titre 2 dilution steps The peripheral staining was less bright than in untreated or formalin treated gono cocci at corresponding dilutions and the reactions were read as 3+

TABLE 2

Staining Reactions of the Reference Strain (GC1) after Treatment with Heat

Treatment of the reference strain	Titres obtained with the reference conjugate (I G-4 (1))		Morphology compared with untreated or formalin treated cells
	3-4 +*	1 2 +*	
Untreated or formalin treated	256	1024	(See Fig 2)
40° C-60° C	256	1024	Unchanged
100° C	128	512	Somewhat changed, see text
120° C	64	256	Somewhat changed, see text

* Degree of staining reactions
3 4+ bright or moderately bright peripheral fluorescence (Fig 2)
1 2+ dull or moderate peripheral fluorescence

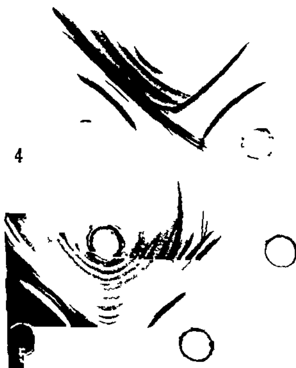


Fig 4 Photograph of a comparative precipitation analysis showing the influence of heat on the ultrasonic (us) treated reference antigen (GC1) Top basin anti GC1 f Left basin GC1 us Right basin GC1 us heated to 100° C for 60 minutes

Fig 5 Photograph of a comparative precipitation analysis before and after the absorption of the reference serum (anti GC1 f) with ultrasonic (us) treated organisms of GC1 Upper left basin anti GC1 f Upper right basin anti GC1 f absorbed with GC1 us Lower basins GC1 us

TABLE 3

Staining Reactions of the Reference strain (GC1) Obtained with the Reference Conjugate (FG-GC1-f) before and after Absorption with Cells of GC1 Treated in Different Ways

Treatment of the FG GC1 f	Dilutions of FG GC1 f									
	1 2	1 4	1 8	1 16	1 32	1 64	1 128	12 6	1 512	1 1021
Untreated	3 4+	3 4+	3 4+	3 4+	3–4+	3 4+	3 4+	3+	1–2+	1+
Absorbed with GC1 f					–	–	–	–	–	–
Absorbed with GC1 us				–	–	–	–	–	–	–
Absorbed with GC1 sup	1 4+	1 3+	1–3+	1–2+	±	–		–	–	–
Absorbed with GC1 sed	1 3+	s s*	s s	s s	–	–		–	–	–
Absorbed with GC1 100° C	3 4+	3–4+	1–4+	1 2+	1+	–	–	–	–	–
Al bed with GC1 120° C	3 4+	3 4+	3–4+	3–4+	3+	1 2+	1+	–	–	–

* s s = spotted staining

TABLE 4

Staining Reactions of the Gonococcal Reference Strain (GC1) in One Step Inhibition Tests

Antı GC1 f used for blocking reactions	Dilutions (final) of the reference conjugate (FG GC1 f)									
	1 2	1 4	1 8	1 16	1 32	1 64	1 128	1 256	1 512	1 1021
0	3 4+	3 4+	3 4+	3 4+	3 4+	3–4+	3–4+	3+	1–2+	1+
Unabsorbed	1 2+	1+	–	–	–	–	–	–	–	–
Absorbed with GC1 f	3 4+	3 4+	3 4+	3 4+	3–4+	3 4+	3+	3+	1+	±
Absorbed with GC1 120° C	3 4+	3 4+	3 4+	3 4+	3+	2 3+	2 3+	1–2+	±	–





Fig 6 Photograph of a comparative precipitation analysis before and after the absorption of the reference serum (anti GC1 f) with formalin (f) treated organisms of GC1 Upper left basin anti GC1 f Upper right basin anti GC1 absorbed with GC1 f Lower left basin normal rabbit serum mixed with GC1 f Lower middle and right basins GC1 us

Fig 7 Photograph of a comparative precipitation analysis before and after the absorption of the reference serum (anti GC1 f) with the sediment (sed) of ultrasonic (us) treated organisms of GC1 Upper left basin anti GC1 f Upper right basin anti GC1 f absorbed with GC1 sed Lower left basin normal rabbit serum mixed with GC1 sed Lower middle and right basins GC1 us

Comparative gel diffusion tests were performed between anti GC1 f and GC1 us before and after heating of the ultrasonic prepared antigen at different temperatures (40°–120° C) Heating of the antigen to 50° C had no influence on the precipitation pattern After heating to 56° C one of the lines in group A was not formed, and after heating to 60° C one line in each of the groups C and D was lacking After heating to 100 C two heavy precipitation lines, one belonging to group C and the other to group D, were formed and in addition five weak lines (Fig 4) Two of these belonged to group A, one to group B and two to group C After heating to 120° C the results were on the whole, the same

6 FA Tests and Gel Diffusion Tests after Absorption of the Reference Conjugate and the Reference Serum

Table 3 shows the staining reactions with GC1 after the FG GC1-f had been absorbed with organisms of GC1 treated in different ways

TABLE 3

Staining Reactions of the Reference strain (GC1) Obtained with the Reference Conjugate (I G-GC1 f) before and after Absorption with Cells of GC1 Treated in Different Ways

Treatment of the I G GC1 f	Dilutions of I G GC1 f									
	1 2	1 4	1 8	1 16	1 32	1 64	1 128	1 256	1 512	1 1024
Untreated	3 4+	3-4+	3-4+	3 4+	3 4+	3-4+	3-4+	3+	1-2+	1+
Absorbed with GC1 f		–	–		–	–	–	–	–	–
Absorbed with GC1 us		–	–	–	–	–	–	–	–	–
Absorbed with GC1 sup	1 4+	1-3+	1-3+	1-2+	±	–	–	–		–
Absorbed with GC1 sed	1 3+	s s*	s s	s s	–	–	–	–	–	–
Absorbed with GC1 100° C	3 4+	3 4+	1 4+	1-2+	1+	–	–	–	–	–
Absorbed with GC1-120° C	3 4+	3 4+	3 4+	3 4+	3+	1-2+	1+	–	–	–

* s s = spotted staining

TABLE 4

Staining Reactions of the Gonococcal Reference Strain (GC1) in One Step Inhibition Tests

Anti GC1 f used for blocking reactions	Dilutions (final) of the reference conjugate (I G GC1 f)									
	1 2	1 4	1 8	1 16	1 32	1 64	1 128	1 256	1 512	1 1024
0	3 4+	3 4+	3 4+	3 4+	3 4+	3 4+	3-4+	3+	1-2+	1+
Unabsorbed	1-2+	1+	–	–	–	–	–	–	–	–
Absorbed with GC1 f	3 4+	3 4+	3 4+	3 4+	3 4+	3 4+	3+	3+	1+	±
Absorbed with GC1 120° C	3 4+	3 4+	3 4+	3-4+	3+	2 3+	2 3+	1-2+	±	–

were formed (Fig 6) After absorption with GC1 sed 10 precipitation lines were seen with the loss of all the lines in group D and some in group A (Fig 7) After absorption with GC1 sup a weak line in group D was left (Fig 8) When anti GC1 f was absorbed with GC1 100° one line in group A and two in group D were lost (Fig 9), and when it was absorbed with GC1 120° the results were in general the same except that only one line in group D was lost

These absorption experiments thus show that all the precipitation lines in group A are eliminated after absorption with GC1 f or with GC1 sup and some of them with GC1 sed GC1 100° and GC1-120° The precipitation lines in group B are wholly eliminated with GC1 sup and partially with GC1 f but not at all with GC1 sed GC1 100° and GC1 120° The precipitation lines in group C are eliminated with GC1 sup but not with GC1 f GC1 sed GC1 100° and GC1 120° The precipitation lines in group D are completely eliminated with GC1 sed almost completely with GC1 f and GC1 100° and partially with GC1 sup and GC1 120°

7 *One Step Inhibition of Staining Reactions*

Table 4 shows the results of one step inhibition tests of the FG GC1 f with the reference serum (anti GC1 f) The latter was used unabsorbed or absorbed with GC1 f or GC1 120°

When the inhibition was performed with the unabsorbed anti GC1 f no 3–4+ reactions were obtained and at the dilution of 1 8 of the FG GC1 f the reaction was completely blocked Anti GC1 f had only a slight inhibiting effect after absorption with GC1 120° and no inhibiting effect at all after absorption with GC1 f

8 *FA Tests and Gel Diffusion Tests with the Reference Strain (GC1) during the Early Phase of Growth and after Prolonged Culture*

FA tests and gel diffusion studied in combination with absorption tests were carried out with the reference strain (GC1) cultured for 12 30 40 and 72 hours The results were compared to those obtained with GC1 cultured for the usual 16–20 hours

In FA tests the gonococci from the 12 hour culture showed the same morphology as those from the 18 hour culture and gave 3–4+ reactions at the same FA titres Similar results were obtained with the 30 and 40 hour cultures but now the smears also contained vague thread like formations (presumably cellular debris) of the same green colour as the stained gonococci (Fig 10) As can be seen from Fig 11 the morphology of the gonococci from the 72 hour culture had almost completely changed The smears also contained granular like formations of the same green colour as ordinary stained gonococci

GC1 was cultured for 40 or 72 hours then treated with formalin or ultrasonic and used for the absorption of the FG GC1 f FA tests were



Fig 8
Photograph of a comparative precipitation analysis before and after the absorption of the reference serum (anti GC1 f) with the supernatant (sup) of ultrasonic (us) treated organisms of GC1 Upper left basin anti GC1 f Upper right basin anti GC1 f absorbed with GC1 sup Lower basins GC1 us



Fig 9
Photograph of a comparative precipitation analysis before and after the absorption of the reference serum (anti GC1 f) with organisms of GC1 heated to 100° C for 60 minutes Upper left basin anti GC1 f Upper right basin anti GC1 f absorbed with GC1 100° Lower left basin normal rabbit serum mixed with GC1 100° Lower middle and right basins GC1 us

After absorption with GC1 f or GC1 us no staining reactions were obtained After absorption with GC1 sup or GC1 sed reactions varying between 1–4+ 1–3+ and 1 2+ were noted at the dilutions of 1 2 1 16 When the conjugate was absorbed with GC1 100 or GC1 120° 3 4+ reactions were obtained but the FA titres were reduced 5 and 3 dilution steps respectively

Comparative gel diffusion tests were performed before and after anti GC1 f had been absorbed with GC1 treated in different ways

After absorption of anti GC1 f with GC1 us no precipitation lines were formed (Fig 5) After absorption with GC1 f five precipitation lines within group C and one line within group B and D respectively

9 FA Tests and Comparative Gel Diffusion Tests after Culturing of GC1 on Different Media

Experiments were performed to determine the effect of culturing the reference strain on chocolate-ascitic agar medium and chocolate-horse-serum agar medium, compared to Difco GC medium

Organisms in smears of GC1 from all the media were stained and no detectable differences at corresponding dilutions of the FG GC1-f were noted No staining reactions were obtained after the FG GC1-f had been absorbed with GC1 cultured on chocolate-ascitic-agar or chocolate-horse serum agar and treated with formalin or ultrasound

Comparative gel diffusion tests with GC1 from all the media gave reactions of identity No precipitation lines were formed when anti-GC1-f was absorbed with ultrasonically treated cells of GC1 cultured on chocolate ascitic agar or chocolate horse-serum agar

10 FA Tests and Gel Diffusion Tests after Subculturing

Subculturing of GC1 colonies, picked at random, was carried out, and the results of FA tests after the 27th and the 102nd subcultures, compared to the 4th, are shown in Table 5

TABLE 5

FA Titres of the Reference Conjugate (FG GC1 f) with the Subcultured Reference Strain (GC1) before and after Absorption with Formalin Treated or Ultrasonic Treated Organisms of Various Subcultures

Number of subcultures of the reference strain (untreated)	FA titres of the reference conjugate (FG GC1 f)						
	Not absorbed	Absorbed with the subcultured reference strain (GC1)					
		Treated w formalin			Treated w ultrasonic		
		4	27	102	4	27	102
4	256			8			8
27	256			4			8
102	256					–	

The staining reactions and the FA titres were the same after 27 or 102 subcultures as after 4 No staining reaction was obtained when the absorbed FG GC1 f was tested with organisms of the absorbing strain of various subcultures After absorption of FG GC1-f with GC1 us or GC1 f of the 102nd subculture FA titres of 4 8 were obtained with organisms of the 4th and 27th subculture

Gel diffusion tests with GC1 us of the 27th and 102nd subculture were performed and the precipitation patterns obtained with anti GC1 f before and after absorption were compared to that obtained with GC1-us of the 4th subculture

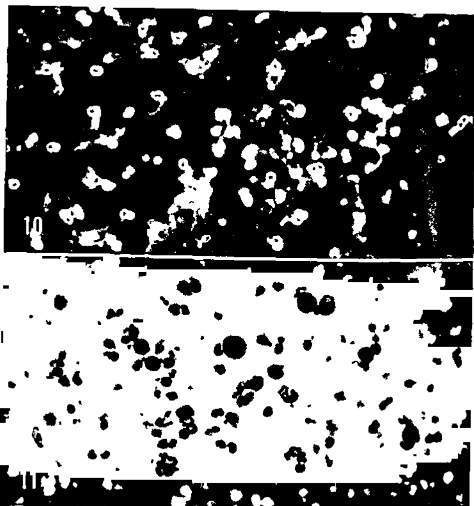


Fig 10 Gonococci of the reference strain cultured for 40 hours and stained with the reference conjugate The morphology moderately changed Antigenic material between the cells has been stained with the conjugate

Fig 11 Gonococci of the reference strain cultured for 72 hours and stained with the reference conjugate The "normal" morphology of most of the cells has been lost, some cells appearing as giant forms Antigenic material between the cells has also been stained

then performed with GC1 cultured for 18, 40 or 72 hours No staining reactions were obtained

Comparative gel diffusion tests with anti-GC1-f and GC1-us from the 18-hour culture gave reactions of identity with the ultrasonic treated antigens of the 12-, 30-, 40- and 72-hour cultures No precipitation lines were obtained when anti-GC1-f was absorbed with ultrasonic treated gonococci of GC1 cultured for 40 or 72 hours and then tested against GC1-us cultured for 18, 40 or 72 hours

and after Absorptions with Formalin Treated or Ultrasonic Treated Organisms

(1 f) before and after absorption

rmalin) used for absorption

12	GC14	GC18	GC20	GC21	GC2	GC5	GC7	GC10	GC15	GC16	GC17	GC19
16	8 16	16	16–32	16–32	8 16	8–16	16	16	8–16	16	8	8–16
8	8	16	16	16	8	16	8	8	8	8	8	18
	–		–	–	–	–		–	–	–	–	–
	–		–	–	–	–	–	–	–	8	–	4
	–		–	–	–	–	–	–	–	–	–	–
	–		–	–	–	–	–	–	–	–	–	–
	–		–	–	–	–	–	–	–	–	–	–
		–	–	–	–	–	–	–		–	–	–
	–	–	–	–	–	–	–	–		–	–	–
–	–		–	–	8	–	–	–	8	8	–	8
	–	–	–	–	–		–	–	–	–	–	–
–	–		–	–	8	–		–		8	–	8
	–	–	–	–	–		–	–		–	–	–
–	–	–			–	–	–	–	–	–	–	
5	8	8	8	16	–	–		–		–	–	–
8	16	8	16	16	–	–		–	–	–	–	–
8	8	16	8	8		–	–	–		–	–	4
4	8	8	8	16		–		–		–		–
8	8	16	16	8	8	8	4	4		8	4	–
6	8	8	16	8	8	–	–		8	–	–	8
8	8	16	8	16	8	–		–	8	–		8
6	16	16	8	18	8	8	4	4	–	8	4	–

FG GC1 I was absorbed with each of the strains GC1-21, which were treated with formalin or ultrasound, and FA tests (cross tests) were then carried out with each of the strains These findings are also summarized in Table 6

Identical results were obtained whether the organisms used for the absorptions were treated with formalin or ultrasound GC1 subcultured 4 27 or 102 times absorbed all the antibodies responsible for the staining reactions of GC2 21 None of these strains were, however, able to absorb all the antibodies responsible for the staining of GC1 but the titre was reduced 3–5 dilution steps Thus, GC1 had in this system the broadest antigenic pattern of the GC strains tested With reference to the absorbing capacity in the present system the strains GC3, 4, 6, 8, 9 11 12 13 14, 18 20 and 21 were the same So were the strains GC5, 7 10 and 17 GC2 differed only slightly from GC16 and GC19 from GC15

TABLE

FA Titres Obtained of the Reference Conjugate (FG GC1 f) with the Gonococcal Strains GC1

Strains tested	FA titres obtained with the reference conjugate									
	No absorption	Strains (treated with ultrasound)								
		GC1 (4 subc)	GC1 (102 subc)	GC3	GC4	GC6	GC8	GC9	GC11	GC12
GC1 (4 subc)	256	–	8	16–32	16	16–32	16	16	16–32	16-32
GC1 (102 subc)	256	–	–	8	0	16	8	16	16	16
GC3	64	–	–	–	–	–	–	–	–	–
GC4	64	–	–	–		–	–	–	–	–
GC6	64	–		–	–	–	–	–	–	–
GC8	64	–	–	–	–	–	–	–	–	–
GC9	64	–	–	–	–	–	–	–	–	
GC11	64	–	–	–	–	–	–	–	–	–
GC12	128	–	–	–	–	–	–	–		–
GC13	64			–		–	–	–	–	–
GC14	128	–	–	–		–	–	–	–	
GC18	64		–	–	–		–	–	–	–
GC20	128			–	–	–	–	–		
GC21	64		–	–	–	–	–	–	–	–
GC2	128	–	–	8	16	16	8	8	8	16
GC5	128	–	–	16	16	16	16	8	16	16
GC7	128		–	16	8	16	16	16	16	16
GC10	64		–	8	8	16	8	16	8	8
GC15	64			16	8	16	8	8	16	16
GC16	64			16	16	16	16	4	16	16
GC17	128			16	8	16	16	8	16	16
GC19	64			16	16	16	16	8	16	16

The same number of precipitation lines were obtained with GC1 us after 27 subcultures as after 4, while one of the two lines nearest the antigen basin seemed to fail with GC1-us of the 102nd subculture When anti-GC1-f was absorbed with GC1-f or GC1-us of subculture 102 and then tested against GC1-us of subculture 4 or 27, a comparative analysis showed that this precipitation line belonged to group D

11 *Comparative FA Tests of the Reference Strain (GC1) and the Gonococcal Strains GC2-21 before and after Absorption of the Reference Conjugate (FG-GC1-f)*

The gonococcal strains GC2-21 were stained with the twofold diluted FG-GC1-f and the FA titres obtained were compared with that obtained with GC1 As can be seen in Table 6, the tested strains GC2-21 gave 3–4+ reactions with the reference conjugate, but for 13 of the strains the FA titres were 2 dilution steps lower and for 7 of them 1 dilution step lower than that of GC1

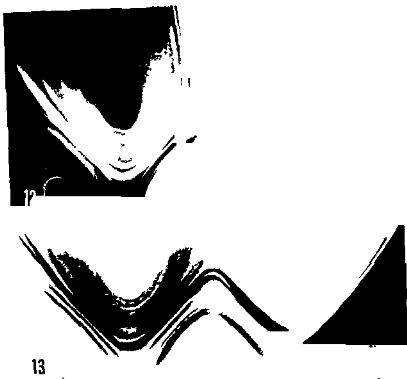


Fig 12 [illegible]

center basin GC1 us

Fig 13 Photograph of a comparative precipitation analysis before and after the absorption of the reference serum (anti GC1 f) with formalin (f) treated organisms of GC12 Upper left basin anti GC f Upper right basin anti GC1 f absorbed with GC12 f Lower left and right basins GC12 us Lower center basin GC1 us

resulted in greater antibody formation than immunization with heat treated gonococci (100° C or 120° C) or fractions (the sediment or the supernatant) of ultrasonic treated gonococci

A good correlation was obtained between the FA titres and the agglutination titres when agglutination was carried out with gonococci treated with formalin This is not quite in accordance with an earlier work (10) by the author, but the differences may be ascribed to improved batches of FITC For example an antigonococcus globulin conjugated with a dye available before 1962 yielded an FA titre of 1 32, while a corresponding globulin conjugated with a dye obtained after 1962 gave FA titres of 1 256 512 and also a more brilliant staining Similar observations concerning FITC-conjugated antistreptococcus globulin were made by *Redys et al* (30)

No correlation could be found between the different absorbing capacities of the strains and their decreased sensitivity to penicillin and resistance to streptomycin.

12 *Comparative Gel Diffusion Tests of the Reference Strain (GC1) and the Gonococcal Strains GC2-21 before and after Absorption of the Reference Serum (anti-GC1-f).*

Comparative gel diffusion tests were carried out between GC1-us and the strains GC2-21, ultrasonically treated, with the use of the anti-GC1-f. This was also absorbed with each of the strains GC2-21, treated with ultrasound, and then tested against GC1-us and the absorbing strain. The results of these tests are summarized in Table 7.

TABLE 7

Number of Precipitation Lines Formed between the Reference Serum and the Gonococcal Strains GC1-21-us before and after Absorption with Ultrasonic Treated Organisms

Strains tested (ultrasonic treated antigens)	Number of precipitation lines formed with the reference serum		
	Before absorption	After absorption with ultrasonic treated organisms of	
		GC1	GC2-21
GC1	15		1 or 2
GC2-21	13 14	n d	-

n d = not done

Twelve strains, GC3, 6, 7, 8, 9, 10, 13, 14, 16, 17, 18, and 21 formed 13 precipitation lines with anti-GC1-f, lacking two lines in the D group, while the other strains formed 14 lines, lacking only one line in the same group When the absorbed anti-GC1-f was tested against the absorbing strain (us) no precipitation lines were formed When it was tested against GC1-us, however, one or two precipitation lines belonging to group D were formed A precipitation pattern typical of such tests can be seen in Fig 12

Ten of the strains GC2-21 were treated with formalin and used for the absorption of anti GC1-f The absorbed and unabsorbed anti-GC1-f was then tested against the absorbing strain (us) and GC1-us A typical precipitation pattern is found in Fig 13, which shows that the absorbed anti-GC1-f formed two additional lines in group D with GC1-us than with GC12-us

DISCUSSION

Four serological techniques—agglutination, complement-fixation, the FA technique, and the double diffusion in-gel technique—were used to estimate the immunological activity of antigonococcal serum from rabbits Immunization with gonococci treated with formalin or ultrasonic

sponsible for the FA staining reactions were absorbed by GC1-f or GC1-us and part of them by GC1-sed, GC1-sup, GC1-100° and GC1-120°. In conclusion, it may be said that the available facts give some evidence that antigenic factors within group D are of importance for the FA staining reaction and that factors within group A may also be of some importance. The antigenic factors within group C seem to have no importance at all for the FA staining and the absorption experiments with intact organisms (GC1-f) and disintegrated organisms (GC1-us) indicated that they are of intracellular origin, which may also be true about the factors within group B.

In this connection it should be pointed out that the poor absorption of precipitins by, for example, GC1-sed, may be due to quantitative differences. Thus, the hyperimmunization of a rabbit with GC1-sed actually produced some antibody factors which belonged to group C. This indicates that GC1-sed contained antigenic factors within group C but that the amount was too small to influence the results.

It should also be pointed out that there may be other antigenic factors of importance for FA staining than those within group A and D but which were not demonstrated in gel diffusion tests. It can be seen in Fig 1 that the ultrasonic treated gonococci contained cellular components of a size that probably did not allow diffusion in agar gel but as they were stained by fluorescent antibodies, they had antigenic properties. The breaking down of these components, probably wall fragments, e g by enzymes, might give further information regarding this point.

It is well known that morphological changes of gonococci occur after culturing for more than two days (42). This was also demonstrated by the FA method. It was of interest to note, however, that after 72 hours' subculturing the bacteria still possessed the ability to absorb all the antibodies taking part in the staining and gel diffusion reactions.

Stokinger, Carpenter & Plack in 1944 (37) performed quantitative agglutination studies of *N gonorrhoeae* and found a significant degree of solubility of certain cell constituents in rabbit serum at physiological pH but the solubility was minimized at pH 5.9. It was shown by gel diffusion tests in the present investigation that, when gonococci were suspended in normal rabbit serum, antigenic factors appeared in the serum both at physiological pH and at pH 5.9 (Figs 6, 7, 9). Most of the factors seemed to be of intracellular origin (Fig 6). This appear- [illegible] be caused by leakage or disintegration [illegible] pronounced at 37° C than at 3–4° C and [illegible] The heating of the serum to 56° C for 30 minutes did not prevent the antigenic factors from appearing in the media. Nor had the addition of polyethylene glycol or dextran 500 (Pharmacia) to the serum any inhibiting effect (1). Similar findings, but less pronounced, were made when gonococci were suspended in PBS. These

It has been argued that the antigen of *N gonorrhoeae* responsible for the staining reaction with FITC-labelled antigonococcus globulins was a heat labile K-antigen of the b-type (12, 14, 20) The present investigation showed that both *thermolabile and thermostable antigenic factors* took part in the staining reaction The FA factors could be partly absorbed by suspensions of gonococci treated by heat at 100–120° C In addition, FITC-labelled antigonococcus globulins which were obtained from rabbits immunized with boiled or autoclaved gonococci gave 3–4+ reactions

In an earlier work (10) by the author strong reactions were obtained between different polyvalent antigonococcus globulin conjugates and 300 gonococcal strains The reference conjugate in the present work and 20 selected gonococcal strains gave similar results, and thus showed that the diagnosis of gonococci may be performed with one species antiserum The Fa titres obtained with these strains were only 1 or 2 dilution steps lower than that obtained with the reference strain (GC1), thus indicating a close antigenic relationship Some antigenic differences were, however, demonstrated by absorption tests, which is in agreement with the findings by *Reyn* (31, 32, 33) and *Wilson* (43) who used complement-fixation and agglutination tests in combination with absorptions In the present investigation, however, only one reference system was used but to undertake an antigenic classification of *N gonorrhoeae* cross reacting experiments should be performed

The experiments utilizing the double diffusion-in-gel technique gave some interesting information With the use of a reference system it was shown *that the reference gonococcal strain contained* at least 15 antigenic factors Due to the difficulty to interpret the individual lines they were classified as belonging to four groups (A D)

The gel diffusion tests showed that both thermolabile and thermostable precipitinogenic factors were represented in all the four groups The precipitinogenic factors within the groups A, B, and C were common to *all the tested gonococcal* strains *One or two factors* within group D of the reference strain GC1 could not be found in the other GC strains, *thus indicating that they may be strain-specific*

Absorption experiments gave no information on the interpretation of the various groups of precipitation lines and their possible relation to FA staining

The antibodies responsible for the precipitation lines within group A were totally absorbed by GC1-f and GC1-sup but only partially by GC1-sed, GC1-100° and GC1-120° The antibodies within group B were completely absorbed by GC1-sup, partially by GC1-f but not at all by GC1-sed, GC1-100° or GC1-120° The antibodies within *group C* were absorbed only by GC1-sup The antibodies within group D were absorbed completely by GC1 sed, almost completely by GC1-f and GC1-100° and partially by GC1-sup and GC1-120° All the antibodies re-

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matters require, however, elucidation by further studies Thus if absorption is performed with intact organisms and the absorption time is varied, for example, 4 hours and 18 hours, somewhat different gel precipitation patterns may be obtained The absorption time does not however, influence the results with the FA technique under these circumstances

SUMMARY

The fluorescent antibody (FA) method and the gel diffusion technique were used to study the antigenic relationship between different strains of *N gonorrhoeae* and the antigens taking part in FA staining and gel precipitation reactions A reference system was selected and used for this purpose

Both heatlabile and heatstable antigenic factors were shown to take part in the FA staining and agar gel precipitation reactions A close antigenic relationship between different gonococcal strains was demonstrated both by the FA method and the gel diffusion technique and the fluorescein isothiocyanate labelled globulin of one species antiserum seems to allow the diagnosis of all gonococcal strains Absorption experiments indicated, however, the occurrence of strain specific antigenic factor(s) A group of antigenic factors demonstrated by the gel diffusion technique were found to be of no importance for the FA staining and a comparison of absorption experiments with intact and disintegrated organisms indicated that they were of intracellular origin

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THE DEMONSTRATION OF *N. GONORRHOEAE* WITH THE AID OF FLUORESCENT ANTIBODIES

4 *Studies by Immunofluorescence and Double Diffusion in-Gel Technique on the Antigenic Relationship between* N gonorrhoeae *and other* Neisseria *Strains*

By

DAN DANIELSSON

Received 10 III 65

The antigenic relationship between *N meningitidis, N catarrhalis* and *N gonorrhoeae* has long been known and has been demonstrated by agglutination, precipitation, and complement-fixation tests (1, 9, 10, 11, 12) *Stokinger, Carpenter & Plack* in 1944 (11) also demonstrated a serological relationship between *N gonorrhoeae* and *N sicca* with the quantitative agglutination technique

Deacon
1961 (6)
and N *g*
anti gono
rhalis This was confirmed by the author (2), who also noted that strains belonging to *N sicca N flava*, and *N flavescens* reacted with antigonococcal globulin conjugates These investigations have been extended in the present work, and the antigenic relationship between *N gonorrhoeae* and other *Neisseria* species has been studied by comparative fluorescent antibody (FA) tests and double diffusion-in gel tests

MATERIALS AND METHODS

Neisseria Strains



tests These strains were isolated at the routine laboratory of the Bacteriological Department in Uppsala and they have been designated with capital U and a number in the tables

Culture

The *Neisseria* strains included above were cultured for 16–20 *hours* on Difco GC medium (7) as described before (2 3)

Serum

An antigonococcus serum obtained from a rabbit immunized with formalin (f) treated organisms of GC1 was used as a reference serum. This antiserum designated anti GC1-f, was used as a reference serum in a previous work (3).

Fluorescent Antibody Procedures

The FITC conjugated globulin of anti GC1 f (FG GC1-f) was prepared as described previously (3) and used as a reference conjugate for the FA tests.
Smears were prepared and FA tests were performed as described elsewhere (2).

Gel Diffusion Tests

The preparation of antigens and treatment with ultrasonic (us) were performed as described previously (3). To obtain lysis of the cells gonococci were treated with ultrasound for 180 sec, meningococci for 240 sec, and the other *Neisseria* strains for 300–420 sec.
The gel diffusion tests were performed as described previously (3) with anti GC1 f as a reference serum and the ultrasonic (us) treated organisms of GC1 (GC1 us) as a reference antigen. This reference system formed at least 15 precipitation lines, which were discussed in an earlier work (3), and classified into 4 groups designated A, B, C and D (Fig. 1).

Absorption Experiments

Gel diffusion tests and FA tests were performed after the reference serum (anti GC1 f) and the reference conjugate (FG GC1 f) had been absorbed with intact organisms treated with formalin or with organisms disintegrated ultrasonically as earlier described (3).
Strains from the National Collection of Type Cultures described above were used for the absorptions.

RESULTS

The results of FA tests between the FG-GC1-f and the gonococcal strains GC1 21 were presented in an earlier work (3). They are, however, included in Tables 1 and 2 for the sake of comparison.

1 *Comparative FA Tests of Different* Neisseria *Strains*

The results of comparative staining reactions between different *Neisseria* strains and the FG-GC1 f are summarized in Table 1.

TABLE 1
FA Titres of N. gonorrhoeae *and other* Neisseria *Strains with the Antigonococcus Reference Conjugate (FG GC1 f)*

Strains tested	FA titres	
	3-4	1-2+
GC1	256	1024
GC2-21	64–128	256–512
MC-A (NCTC 10025)	32	128
MC-B (NCTC 10026)	16	64
MC C (NCTC 8554)	32	128
MC D (NCTC 8557)	4	8
N *catarrhalis* (NCTC 3622 & U1)	<2	8
N *sicca* (NCTC 4591 U2 3)	<2	4
N *sicca* (U1)	<2	<2
N *flava* (NCTC 4590 U1 2)	<2	4–8
N *flavescens* (NCTC 8263 U1)	<2	4

(i) *N meningitidis* The tested meningococcal strains gave 3–4 + reactions with the FG GC1 f but the FA titres were 3–5 dilution steps lower than that of GC1 and 1–4 dilution steps lower than those of the gonococcal strains GC2 21

(ii) *N catarrhalis sicca, flava and flavescens* No 3–4 + reactions were obtained with these strains Weak reactions, estimated as 1–2 +, could be seen but they disappeared when the conjugate was diluted more than 1 8 One strain of *N sicca*, (U 1), did not react at all with the conjugate

2 *Staining Reactions and FA Titres Obtained after Absorptions of the Reference Conjugate*

The staining reactions and the FA titres obtained after absorption of FG GC1 f are summarized in Table 2 The same results were arrived at whether the organisms used for absorptions were treated with formalin or ultrasound

(i) *Absorption with* N meningitidis *group A or group* C The absorp*tion of the FG GC1 f* with group A or group C meningococci resulted in a lowering of the FA titres of GC1 21 with 3 4 dilution steps The 3–4 + *reactions* with meningococci were eliminated but weak reactions with *heterologous* meningococci still occurred After these absorptions the

a N flava, and *N*

N flava The absorp

tion of the FG GC1 f with these strains had no influence on the FA *titres* of N *gonorrhoeae* or N *meningitidis* The weak cross *reactions* with N *catarrhalis* were no longer visible after absorption of the conjugate with this strain but were still there in tests with N *sicca* N *flava* and N *flavescens* Corresponding results were obtained in absorption tests with N sicca and N *flava*

3 *Comparative Gel Precipitation Tests before and after the Absorption of the Reference Serum (anti GC1 f)*

i N meningitidis The ultrasonic treated organisms of N *meningitidis* group A B C or D formed *10 or 11* precipitation lines with anti GC1 f (Figs 2 and 3) Compared with the precipitation *lines of* the reference system (anti GC1 f and GC1 us) they belonged to the groups A B and C No lines were formed in *group D*

A typical precipitation pattern after the absorption of anti GC1 f with intact meningococci is shown in Fig 2 The absorbed anti GC1 f formed three lines with GC1 us *(one of them somewhat weakened)* within group D The pr*ecipitation lines* within group C were on the whole unchanged and in the *original* plate one or two weak lines in group B were observed All the other *lines were lacking*

A typical precipitation after absorption with *meningococci, disinte*

Serum

An antigonococcus serum obtained from a rabbit immunized with formalin (f) treated organisms of GC1 was used as a reference serum This antiserum designated anti GC1 f was used as a reference serum in a previous work (3)

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Gel diffusion tests and FA tests were performed after the reference serum (anti GC1 f) and the reference conjugate (FG GC1 f) had been absorbed with intact organisms treated with formalin or with organisms disintegrated ultrasonically as earlier described (3)
Strains from the National Collection of Type Cultures described above were used for the absorptions

RESULTS

The results of FA tests between the FG GC1 f and the gonococcal strains GC1 21 were presented in an earlier work (3) They are however included in Tables 1 and 2 for the sake of comparison

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TABLE 1

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Strains tested	FA titres	
	3+	1 2
GC1	256	1024
GC2 21	64 128	2o6 512
MC A (NCTC 10025)	32	128
MC B (NCTC 10026)	16	64
MC C (NCTC 8554)	32	128
MC D (NCTC 8557)	4	8
N *catarrhalis* (NCTC 3622 & U1)	<2	8
N *sicca* (NCTC 4591 U2 3)	<2	4
N *sicca* (U1)	<2	<2
N *flava* (NCTC 4o90 U1 2)	<2	4–8
N *flavescens* (NCTC 8263 U1)	<2	4

al n Treated or Ultrason c Treated Organisms of D fferent Neisseria *Strains*

u a e af er abso p ons w h

N a arrh NCTC 3622		N cca NCTC 4 91		N fla a NCTC 4 30	
3-4 +	1 2 +	3-4 +	1 2 +	3-4 +	1 2 +
256	1024	256	1024	256	1024
64 128	128–512	64–128	128–512	64–128	128–512
32	64	32	64	32	64
16	32 64	16	32 64	16	32 64
32	64–128	32	64–128	32	64–128
4	16	4	16	4	16
	<2		2 4		2 4
	<2		2 4		2 4
	2 4		<2		2 4
	2 4 or oc s s		2 4 or		2 4 or
	2 4		2 4	–	<2
	2 4		2 4		<2
	2 4		2 4		2 4
	2 4		2 4		2 4

or N *flavescens* gave the same results A typical precipitation pattern is seen in Fig 5 which shows that a few precipitation lines in group A were not formed between the absorbed anti GC1 f and GC1 us while the lines in the other groups remained intact

A typical precipitation pattern after absorption with N *sicca* N *flava* or N *flavescens* disintegrated with ultrasound is seen in Fig 6 which shows that the absorbed anti GC1 f formed no precipitation lines with the absorbing strain but three lines with GC1 us in group C and three l nes in group D

DISCUSSION

In the present work the antigenic relationship between N *gonorr hoeae* and other *Neisseria* species was studied by the FA method and the gel diffusion technique with the use of a reference system which c nsisted of a gonococcus strain and its corresponding antiserum ob tained from r rbl it This reference system was used by the author in a prev us work (3) in a serological study of gonococcal strains

The FITC conjugated antigonococcus globulin gave strong cross reactions with meningococci thus confirming earlier findings (2 5 6) The comparative FA tests in the present work showed that most of the m ningococcal strains were stained by the antigonococcus conjugate at s mewhat lower titres than gonococci

TABLE

FA Titres Obtained with the Reference Conjugate (FG GC1 f) after Absorption w

Strains tested	FA titres of the reference			
	N mening A NCTC 10025		N mening C NCTC 8554	
	3-4 +	1 2 +	3-4 +	1 2 +
GC1	32	64–128	32	64 128
GC2-21	8–16	32 64	8 16	32 64
MC–A (NCTC 10025)	–	<2	–	2-4
MC–B (NCTC 10026)	–	2–4	–	2-4
MC–C (NCTC 8554)	–	2–4	–	<2
MC–D (NCTC 8557)	–	2	–	2
N *catarrh* (NCTC 3622)	–	2–4 oc s s *	–	2 4 oc s s
N *catarrh* (U 1)	–	2 4 oc s s	–	2 4 oc s s
N *sicca* (NCTC 4591)	–	2 4 or – 2 4	–	2 4 oc s s
N *sicca* (U 1, 2, 3)	–	oc s s oc s s	–	2 4 or oc s s
N *flava* (NCTC 4590)		2–4 oc s s	–	2 4 oc s s
N *flava* (U 1 2)	–	2 4 oc s s	–	2-4 oc s s
N *flavesc* (NCTC 8263)	–	2 4 oc s s	–	2 4 oc s s
N *flavesc* (U 1)	–	2 4 oc s s	–	2 4 oc s s

* oc s s = occasional spotted staining

grated with ultrasound, is seen in Fig 3, which shows that the absorbed anti GC1-f formed no precipitation lines with the absorbing strain, but one heavy line with GC1-us in group C and three lines (one of them somewhat weakened) within group D No precipitation lines were formed in group A and B

(ii) *N catarrhalis* The ultrasonic treated organisms of *N catarrhalis* formed two or three weak precipitation lines with anti-GC1-f, probably belonging to group A and visible in the original plate (Fig 4)

A typical precipitation pattern after absorption with intact organisms of *N catarrhalis* is seen in Fig 4, which shows that a few precipitation lines in group A were not formed between the absorbed anti-GC1-f and GC1-us while the other lines remained intact After absorption with ultrasonic treated organisms no precipitation line was formed with the absorbing strain A few lines in group A of the reference system were lacking while the other lines were unchanged

(iii) *N sicca, N flava and N flavescens* The ultrasonic treated organisms of *N sicca, N flava,* and *N flavescens* formed 8–9 precipitation lines with anti-GC1-f (Figs 5 and 6) which belonged to the groups A, B and C of the reference system No precipitation lines were formed in group D

Absorption of anti-GC1-f with intact organisms of *N sicca, N flava*







Fig 4 ter the (NCTC Upper *rmalin* and right

Fig 5 after the CTC 4590) Jpper right basin anti GC1 f absorbed with N *flava* treated with *formalin* Lower center basin ultrasonic treated antigen of GC1 Lower left and right basins ultras nic treated antigen of N *flava*

Fig 6 Photograph of a comparative precipitation analysis before and after the absorption of the reference serum (anti GC1 f) with N *sicca* (NCTC 4591) treated with *ultrasound* Upper left basin anti GC1 f unabsorbed Upper right basin anti GC1 f absorbed with N *sicca* treated with ultrasound Lower center basin ultrasonic treated antigen of GC1 Lower left and right basins ultrasonic treated antigen of N *sicca*

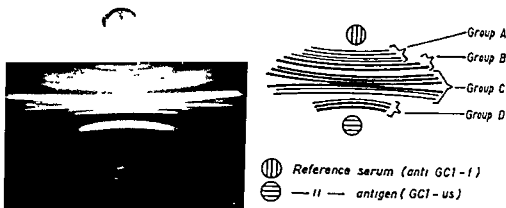


Fig 1

Photograph (A) and drawing (B) of the precipitation pattern formed by the reference system Upper basin reference serum (anti-GC1-f) Lower basin ultrasonic (us) treated antigen of the reference strain GC1 (GC1-us)

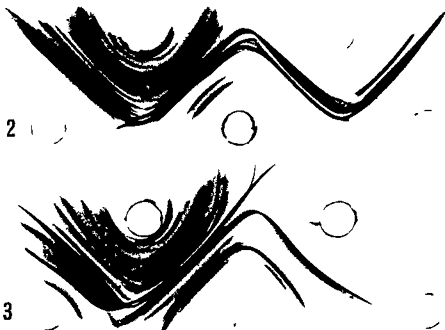


Fig 2 Photograph of a comparative precip ... the
absorption of the reference serum (ant
(NCTC 10025) treated with *formalin*
sorbed Upper right basin anti GC1 f
treated with *formalin* Lower center basin ultrasonic treated antigen of GC1
Lower left and right basins ultrasonic treated antigen of N *meningitidis*
group A

Fig 3 Photograph of a comparative precipitation analysis before and after the absorption of the reference serum (anti GC1 f) with N *meningitidis* group C (NCTC 8554) treated with *ultrasonic* Upper left basin anti GC1 f unabsorbed Upper right basin anti GC1 f absorbed with N *meningitides* group C treated with *ultrasound* Lower center basin ultrasonic treated antigen of GC1 Lower left and right basins ultrasonic treated antigen of N *meningitidis* group C

ported by the findings in this work There is thus some evidence that a few antigenic factors in group A may be of minor importance for the FA staining The antigenic factors in the D group appear to represent some main antigens responsible for the FA staining of gonococci, confirming the results arrived at in the previous work (3) The results of the absorption tests also showed that the antigenic factors in group D and one in group C were speciesspecific In a previous work it was also found that one antigenic factor in group D might be strain specific

As pointed out in a previous work (3), ultrasonic treated organisms of gonococci might contain antigenic components that are of importance for the FA staining but of such a size that they would not diffuse in agar gel This could also be true of the meningococci

In the present investigation, the serological studies were carried out with the use of a gonococcus reference system only In order to obtain more information about the antigenic relationship between various Neisseria species, cross reacting experiments must be performed and such work is now being undertaken

SUMMARY

The antigenic relationship between *N gonorrhoeae* and other *Neisseria* species was studied by comparative fluorescent antibody (FA) tests and gel diffusion tests with the use of a gonococcus reference system

Strong reactions were obtained between fluorescein isothiocyanate (FITC) labelled antigonococcus globulin and meningococci but only weak reactions at low dilutions of the conjugate with so called apathogenic *Neisseria* A conjugate specific for only gonococci can be obtained by absorption From a practical point of view this does not seem to be necessary for the diagnosis of genito urinary gonorrhoea

Gel diffusion tests showed a relatively close antigenic relationship between gonococci and meningococci and certain apathogenic *Neisseria* strains Most of these antigens were of *intracellular origin* The gel diffusion tests also demonstrated the occurrence of species-specific antigens in gonococci and some of these were of importance for the FA staining

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The strong cross reactions with meningococci might be a source of error if untreated FITC-labelled antigonococcus globulins are used for the diagnosis of gonococci The cross-reactions could, however, be eliminated by homologous absorption with intact organisms treated with formalin or with organisms disintegrated with ultrasound From a practical point of view a mistake in the diagnosis is unlikely since meningococci are extremely rare in the genito-urinary tract In a series of 37 000 specimens from men and women no meningococcal strain was isolated from this region by cultural procedures (4) Similar experience has been reported from other laboratories (8)

The antigonococcus globulin conjugate used in the present work gave only weak reactions at low titres with the so-called apathogenic *Neisseria species* Two of the tested strains, one belonging to *N catarrhalis* and the other to *N sicca*, were isolated from the cervix and the urethra of two women, and they were easily distinguished from gonococci by FA tests with the use of a diluted antigonococcus globulin conjugate

The close antigenic relationship between gonococci and meningococci, demonstrated in the present work with the FA method, and by others with different serological techniques (1, 9, 10, 11, 12), was confirmed by gel diffusion tests An antigenic relationship between gonococci and other *Neisseria* species was also established With the reference system used, *N sicca, N flava* and *N flavescens* resembled meningococci, while *N catarrhalis* showed a more remote relationship

The findings by the gel diffusion technique also threw some light on the antigenic components that may be responsible for the FA staining and the antigens that may be species specific

Absorption with intact meningococci eliminated the FA reactions with these organisms and lowered the FA titres with gonococci It eliminated the precipitation lines in group A of the reference system and weakened the lines in group B, but left the lines in group C and D untouched Absorption with disintegrated meningococci gave the same results in the FA tests In gel diffusion tests, however, a corresponding absorption eliminated all precipitation lines in group A and B, but left the lines in group D and one line in group C untouched Absorption with intact organisms of a so-called apathogenic *Neisseria* strain, for example *N sicca*, eliminated the weak cross reactions with these organisms but did not influence the FA titres with gonococci It eliminated a few precipitation lines in group A of the reference system but left the other lines untouched Absorption with disintegrated organisms of *N sicca* gave the same results in the FA tests In gel diffusion tests it eliminated all the lines in group A and B of the reference system leaving all the lines in group D and three lines in group C untouched In a previous work (3) it has been shown that the C-lines, and probably also the B-lines, are likely to represent intracellular substances without importance for the FA staining, and this is also sup-

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TRIC AGENTS ISOLATED IN DENMARK

I *Isolation of the TRIC Agent from Inclusion Blennorrhoea*

By

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Received 18 xi 64

Intensive efforts to cultivate the causative agents (TRIC) of trachoma and the related inclusion conjunctivitis were carried out for years in many laboratories without definite isolation of the agent as achieved in series or in quantity Not until 1957 in Peking *T ang et al* (1) were able to give a convincing report of the isolation of 3 elementary body strains from patients with trachoma These isolations were carried out by inoculation of conjunctival scrapings into the yolk sac of chick embryos and by use of high concentrations of streptomycin in preventing bacterial growth Shortly after *Collier & Sowa* (2) confirmed the results by the Chinese workers and in the following years the TRIC agent has been isolated from trachoma patients in a number of countries (3)

When applying the same technique using scrapings from cases of inclusion blennorhoea (inclusion conjunctivitis in the newborn) *Jones et al* (4) in London and later *Hanna et al* (5) in San Francisco were able to isolate elementary body strains from such patients Morphologically and antigenically these agents were indistinguishable from the elementary body strains isolated from trachoma patients However, the isolation of the TRIC agent from inclusion blennorrhoea has been found more difficult than isolation of the agent from trachoma patients despite the ease with which inclusion bodies can be demonstrated in the former disease (3)

During the last 2 years 5 cases of inclusion blennorrhoea have been observed at the eye clinic Rigshospitalet Copenhagen The present paper describes the isolation of the TRIC agent from 3 of the 5 patients with inclusion blennorrhoea and a similar agent from the cervix of one of the mothers A brief report has previously been published in this journal (6)

The valuable technical assistance by Miss *E Christensen* and Miss *E Nohr* is gratefully acknowledged

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Toxicity tests Young 3 week old albino Swiss mice were lightly anaesthetized 1 th a 10–30 per cent broth saline suspen

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respectively In add tion the medium was further enriched with 3 per cent yeast autolysate

RESULTS

Ocular TRIC Agent Infection of the Newborn

Clinical examination A typical clinical picture of inclusion blennorrhoea infection was seen in all 5 newborn examined As indicated in Table 1 all patients had their first symptom of infection between 6 and 9 days after birth The clinical picture was characterized by a profuse purulent discharge pronounced palpebral oedema heavy hyperaemia and infiltration of the conjunctival epithelium and in most cases these symptoms were followed by pseudomembrane formation over the conjunctival surface No corneal involvement was seen in any case

Bacteriological cultures From each patient repeated tests for growth of bacteria from the affected conjunctiva were carried out At no time was any pathogenic bacteria isolated when tested upon blood agar plates or in Stewart s medium

Microscopical examination of conjunctival smears Giemsa stained smears of conjunctival scrapings revealed the presence of cytoplasmic inclusion bodies in all the 5 patients examined (Table 1) These inclusion bodies were found in different numbers in various scrapings, with 1 to 2 and up to more than 25 in a single scraping When treatment with chloramphenicol was discontinued an increase in the number of inclusion bodies could be observed in conjunctival scrapings

Isolation of elementary body strains From 3 out of 5 newborn an elementary body strain was isolated after inoculation of conjunctival scrapings into the yolk sac of the chick embryo From each positive patient repeated isolates were obtained from specimens collected on

MATERIALS AND METHODS

Patients All patients were examined at the Out patients clinic of the Department of Ophthalmology University of Copenhagen The clinical diagnosis of inclusion blennorrhoea was made among newborn referred to the eye clinic for a purulent conjunctivitis from the Departments of Obstetrics and Gynecology A and B University of Copenhagen

Specimens Scrapings of epithelial cells were taken with a platinum spatula from conjunctiva of the affected eye Scrapings inal area of the cervical c s using a shallow as intended to collect only the more super prevent bleeding No anaesthetic was used. The obtained material was subjected to microscopic examination and attempts of isolation of the actiologic agents

Microscopic examination of epithelial scrapings During each clinical examination scrapings were smeared on a glass slide and fixed with absolute methyl alcohol for 15 minutes then stained for 1 hour with stock Giemsa (Merck) diluted 1 25 n distilled water After staining smears were washed for 10 seconds in 95 per cent ethyl alcohol to wash off excess stain Smears were then examined microscopically first under low power (8 × ocular and 40× objective) and subsequently under oil immersion (8 × ocular and 90 × objective) Each slide was searched at least 30 minute ide as negative The sole criterio of one or more inclusion bodies helial cells and contained a varying number of elementary bodies in each inclusion body The general cytological picture was recorded with special emphasis upon number of leucocytes and monocytes

Technique of egg isolation Each scraping for isolation was immediately collected in 1 ml broth saline (tryptose phosphate bouillon) containing streptomycin sulphate 10 mg ml polymyxin B sulphate 0 1 mg/ml and neomycin 0 5 mg/ml The collected specimens in broth were kept on ice bath for 2 3 hours until brought to the laboratory The suspensions were then kept at +4° C for about 2 hours and then inoculated in 0 4 ml to 0 5 ml amounts into the yolk sacs of chick embryos which had been incubated at 37° C for 6 8 days Subsequently the eggs were incubated at 35° C and candled at least once daily thereafter If the embryos died within 48 hours f inoculation the eggs were discarded The yolk sacs of surviving embryos were harvested after 11 13 days and subjected to at least 2 additional blind passages before regarding an isolation attempt as unsuccessful

Eggs inoculated with human material in the first passage occasi nally died in the period from the 8th to 13th day after inoculation Embryos which survived the 11–13th day after inoculation were killed by chilling at —20° C for half an hour Impression smears of yolk sac membranes of dead and sacrificed embryos were stained by Giemsa s method and examined microscopically for elementary bodies under oil immersion When present these particles were seen as individual intense bluestained elementary bodies scattered across the smears Harvested yolk sacs were weighed shaken with glass beads in a screw cap vial for 1 2 minutes and made into 20 per cent suspensions with streptomycin containing broth suspension (2 4 mg ml) Approximately 0 5 ml was then inoculated into the yolk sac of 6 8 day old embryonated eggs The membranes used for passage were in most processed and inoculated into a new passage the same day as harvested otherwise stored at —60° C until used for new passage

Infectivity titrations The egg LD50 of established elementary body isolates were estimated by inoculation of groups of 6 8 eggs with 10 fold dilutions of yolk sac suspension recording deaths which occurred between 3rd and 12th day of incubation and checking presence of elementary bodies in stained smears of yolk sacs from dead embryos Fifty per cent death endpoints (LD50) were calculated by the method of

1

By this method a thermostabl

and followed by acetone precipitati n Controls were prepared from normal yolk sac suspensions

consecutive days All these elementary body strains had morphological and tinctorial properties identical to those of the agents of the psittacosis lymphogranuloma venereum (LGV)-trachoma group

In Table 1 various data pertaining to the isolation experiments are listed The first scraping for isolation from each patient was collected between 7 and 29 days after onset of eye symptoms All patients had been treated ocularly with chloramphenicol from 1 to 3 weeks previous to collection of the first specimen In addition, one to three more attempts of isolation were carried out during the following 2 to 7 days in which period no treatment was given However, no difference in the rate of positive isolates could be demonstrated between specimens collected before or after the treatment was discontinued As mentioned previously the scrapings were usually kept at +4° C for 2 to 4 hours before inoculated into the chick embryo In TRIC containing specimens it was possible also to re-isolate the agent from scrapings kept at 60° C from 8 days up to 6 months Free elementary bodies were in most cases seen in yolk sac smears in the first or second yolk sac passage and specific death of inoculated embryos occurred normally in the same or the following yolk sac passage No essential difference in time of death or findings of free elementary bodies was observed whether scrapings had been kept at +4° C for a few hours or at —60° C up to 6 months

From 2 of the 5 newborn under investigation neither free elementary bodies nor specific death of the embryos occurred in spite of repeated isolation attempts As the eggs used during this experimental period were partially insusceptible (10), additional conjunctival scrapings as well as material from previously inoculated yolk sacs were kept at —60° C for 5 to 6 months When subsequently inoculated into eggs, which at this time again had a normal susceptibility, the results were still negative Some of these inoculates have been through 5 to 6 blind yolk sac passages

TABLE 2

Designation of TRIC Agent Isolates Statens Seruminstitut Copenhagen

Patient	Source of isolate	Date of Yolk sac inocul	Designation*
Baby GC	TRIC agent ophthalmia neonatorum	Jan 1963	TRIC/ /DK/SS–1 ON
Baby IP	TRIC agent ophthalmia neonatorum	Oct 1963	TRIC/ /DK/SS–2 ON
Baby LP	TRIC agent ophthalmia neonatorum	June 1964	TRIC/ /DK/SS–3 ON
Mrs LP	TRIC agent cervicitis in mother to baby LP	June 1964	TRIC/ /DK/SS 4 GCx§

* Designation according to recommendations of Trachoma Group International Congress of Microbiology Montreal 1962 (19)
§ GCx Genital isolate obtained from the cervix

Genital TRIC agent infection of the mothers Cervical scrapings were obtained from 3 of the 5 mothers with inclusion positive babies Microscopic smears of these scrapings disclosed a cytology very much similar

TABLE 1

Data Pertaining to Attempts of TRIC Agent Isolation from 5 Cases of Inclusion Blennorrhoea

Patient (baby)	Age at onset (days)	Specimens collected day after onset	Treatment prior to sampling	Pathogenic bacterial organisms	Inclusion bodies	Storage (C) of specimens before egg inoculation		Isolation experiments			
								No of egg pass for isol		Yolk sac passages total	Infective titer (egg LD50 ml) final
								FB*	Death		
GC	8	24	chloramph	0†	+§	—60°	8 days	2	2	9	
		26	none	0	++	+ 4°	2 hrs	2	2	25	6 4 ‖
I P	7	7	chloramph	0	+	+ 4°	4 hrs	2	2	2	
		9	none	0	++	+ 4°	2 hrs	1	2	12	6 1
		9	none	0	++	—60°	2 mths	1	2	3	
		11	none	nd°	+++	+ 4°	3 hrs	1	1	2	
		11	none	nd	+++	—60°	6 mths	2	2	4	
U P	9	29	chloramph	0	++	+ 4°	2 hrs	2	3	10	6 2
		34	none	nd	++	+ 4°	3 hrs	2	2	5	
L GP	6	11	chloramph	0	+	+ 4°	3 hrs	0	0	5	
		12	none	0	+++	—60°	8 days	0	0	5	
		13	none	nd	++	—60°	6 mths	0	0	3	
		15	none	nd	++	+ 4°	3 hrs	0	0	6	
		15	none	nd	++	—60°	6 mths	0	0	4	
KA	9	12	chloramph	0	+	+ 4°	3 hrs	0	0	4	
		12	chloramph	0	+	—60°	5 mths	0	0	4	
		18	none	0	+++	+ 4°	3 hrs	0	0	5	
		18	none	0	+++	—60°	6 mths	0	0	3	

* FB = Free elementary bodies † 0 = Negative
§ + = 1-5 inclusion bodies ++ = 5-10 inclusion bodies +++ = >10 inclusion bodies
° nd = Not done ‖ = Negative log of dilution resulting in 50 per cent death of eggs

consecutive days All these elementary body strains had morphological and tinctorial properties identical to those of the agents of the psittacosis lymphogranuloma venereum (LGV)-trachoma group

In Table 1 various data pertaining to the isolation experiments are listed The first scraping for isolation from each patient was collected between 7 and 29 days after onset of eye symptoms All patients had been treated ocularly with chloramphenicol from 1 to 3 weeks previous to collection of the first specimen In addition, one to three more attempts of isolation were carried out during the following 2 to 7 days in which period no treatment was given However, no difference in the rate of positive isolates could be demonstrated between specimens collected before or after the treatment was discontinued As mentioned previously the scrapings were usually kept at +4° C for 2 to 4 hours before inoculated into the chick embryo In TRIC containing specimens it was possible also to re isolate the agent from scrapings kept at —60° C from 8 days up to 6 months Free elementary bodies were in most cases seen in yolk sac smears in the first or second yolk sac passage and specific death of inoculated embryos occurred normally in the same or the following yolk sac passage No essential difference in time of death or findings of free elementary bodies was observed whether scrapings had been kept at +4° C for a few hours or at —60° C up to 6 months

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Baby UP	TRIC agent ophthalmia neonatorum	Oct 1963	TRIC/ /DK/SS-2/ON
Baby UP	TRIC agent ophthalmia neonatorum	June 1964	TRIC/ /DK/SS 3 ON
Mrs. UP	TRIC agent cervicitis in mother t baby UP	June 1964	TRIC/ /DK/SS 4 GCx§

* Designation according to recommendations of Trachoma Group International Congress of Microbiology Montreal 1962 (19)
§ GCx Genital isolate obtained from the cervix

Genital TRIC agent infection of the mothers Cervical scrapings were obtained from 3 of the 5 mothers with inclusion positive babies Microscopic smears of these scrapings disclosed a cytology very much similar

to that of the conjunctiva of the babies, but in none of these scrapings could any inclusion bodies be demonstrated However, an elementary body strain was isolated from one of the mothers This woman was the mother of one of the 3 babies (Mrs UP, Table 2) who yielded a positive isolate Free elementary bodies in yolk sac smears of this isolate were first detected in the 3rd passage and death of the embryos occurred in the following passage Only specimens kept at +4° C were tested

Serological identification All isolates could be shown to react as a common group antigen of the psittacosis-LGV-trachoma group of agents A CF-antigen titre of 1 125 to 1 512 was measured

Pathogenicity of monkeys Inoculation by the conjunctival route of cynomolgus monkeys (9) with the isolated strains caused a typical follicular conjunctivitis of the monkey eye When stained with Giemsa, conjunctival scrapings revealed many inclusion bodies and it was possible to reisolate the agent repeatedly in yolk sacs of the chick embryo

Laboratory infection Following handling of one of the two isolates SS–1/ON and SS 2/ON a laboratory worker developed a follicular conjunctivitis with numerous inclusion bodies in epithelial cells of the conjunctiva A reisolation of the agent from the conjunctiva was successful

Pathogenicity of mice In tests for toxicity in mice of the isolated strains, a 10 30 per cent suspension of heavily infected yolk sac membranes inoculated intravenously regularly killed the animals Gross examination of the latter revealed congestion of the lungs and haemorrhagic lesions of the small intestine Weanling mice were not killed by the isolated elementary body strains after intracerebral inoculation

Tests for pleuropneumonia-like organisms None of the isolated strains contained pleuropneumonia-like organisms when tested both under aerobic and anaerobic conditions

DISCUSSION

In 3 out of 5 cases an isolate has been obtained from a newborn with inclusion blennorrhoea When subjected to various laboratory tests the isolated agents were all indistinguishable morphologically from the elementary bodies of the psittacosis-LGV trachoma group of agents In addition they all contained the heat stable complement fixing antigen common for this group of agents The biological similarities of the isolates to the trachoma-inclusion conjunctivitis (TRIC) group were confirmed when inoculated onto the monkey conjunctiva and also by the fact that one of the isolates caused a typical inclusion conjunctivitis infection in a laboratory worker These isolations of a TRIC agent from cases of inclusion blennorrhoea are in agreement with the results reported by *Jones et al* (4) from London and *Hanna et al* (5) in San Francisco As far as the author is aware, the TRIC agent has not pre-

viously been isolated in the Northern part of Europe from either trachoma or inclusion conjunctivitis The isolation of the TRIC agent from the genital tract of one of the mothers whose baby yielded a positive isolate, confirms and extends the results by *Jones et al* (11)

In all cases of successful isolation from a single scraping of the baby eye, the recovery of the elementary body strain was obtained in the first or the second egg passage Usually the agent was lethal for chick embryos already in the same or next egg passage whenever visible elementary bodies had been found in impression smears of harvested yolk sac membranes Similar observations have been made by *Sowa et al* (12) and *Murray et al* (13) when attempts of egg isolation of the TRIC agent were carried out from trachoma patients Thus *Sowa* (12) found that a positive isolate from trachoma patients in most cases would appear in the first or second egg passage if the agent was present in Giemsa stained scrapings from the affected conjunctiva and surviving eggs were sacrificed not until 11 to 13 days of inoculation The data presented in this study thus seem to indicate that strains of inclusion blennorrhoea are similar to trachoma strains in terms of ability to kill chick embryos in their first egg passages

In the present study conditions for recovery of the TRIC agents were to a large extent uniform throughout the study period The collection and handling of material for isolation was carried out under similar conditions All patients had an active clinical disease when the first isolation attempt was made Furthermore, all patients under examination had previously been treated ocularly with chloramphenicol from 1 to 4 weeks From experiments in eggs it has been shown that chloramphenicol does have a moderate antimicrobial effect upon strains of TRIC agents (14) In accordance, all treatment was temporarily withheld, while conjunctival scrapings were examined for inclusion bodies and specimens for egg isolation were collected However, the rate of recovery of the aetiological agent seemed to be identical whether the patient had been treated with chloramphenicol or not, although the number of inclusion bodies increased in conjunctival scrapings when the treatment was discontinued Thus the recovery rate of the inclusion blennorrhoea agent seemed to be independent of the treatment with chloramphenicol and the number of inclusion bodies present in patient's conjunctival scrapings A similar lack of correlation between the number of inclusion bodies and the recovery of the agent in eggs were shown by *Hanna et al* (15) among children with a typical trachoma infection In that study none of the affected children yielding a positive isolate had any inclusion bodies in conjunctival scrapings, whereas an adult with a large number of inclusion bodies failed to yield any isolate

The viability of the trachoma elementary bodies in conjunctival scrapings has been shown to persist at —60° C for many months (16) *Hanna et al* (17) reported the isolation of TRIC agents from either fresh or frozen clinical specimens of inclusion blennorrhoea However,

freezing and storage necessitated additional blind passages in eggs In the present study the rate of recovery in eggs of the TRIC agents from inclusion blennorrhoea was not impaired by storage of conjunctival scrapings at —60° C from 8 days up to 6 months Isolates were obtained from both fresh and frozen specimens collected on the same day and in these cases no delay in recovery from eggs occurred

The reason for the failure to obtain an isolate from 2 of the 5 patients is obscure It was first thought that the unsuccessful isolation in these cases could possibly be explained by a partial egg insusceptibility which was observed in the eggs during part of the experimental period Such a fluctuation in susceptibility of embryonated eggs had been suspected to be perhaps one of the most important factors for successful isolation of the TRIC agent (18) However, this assumption was not supported when the tests were repeated at a later date in eggs of normal susceptibility with material which had been kept frozen at —60° C Although repeated blind passages were carried out no isolates were obtained from these 2 cases

SUMMARY

This paper reports the isolation of the TRIC (trachoma-inclusion conjunctivitis) agent from 3 out of 5 Danish babies with inclusion blennorrhoea Inoculation of conjunctival scrapings into the yolk sacs of embryonated hen egg was employed Each of the 3 isolated strains was repeatedly isolated from the individual patients In addition one TRIC agent was isolated from the cervix of one of the mothers whose baby yielded a positive isolate

Only one or two blind passages were required for recovery of any one strain No loss in viability could be demonstrated when conjunctival scrapings were kept in broth saline at —60° C for up to 6 months The data reported here on isolation of TRIC agents in the yolk sacs of chick embryos from the newborn with inclusion blennorrhoea are similar to those described for isolation of TRIC agents from trachoma patients in other countries

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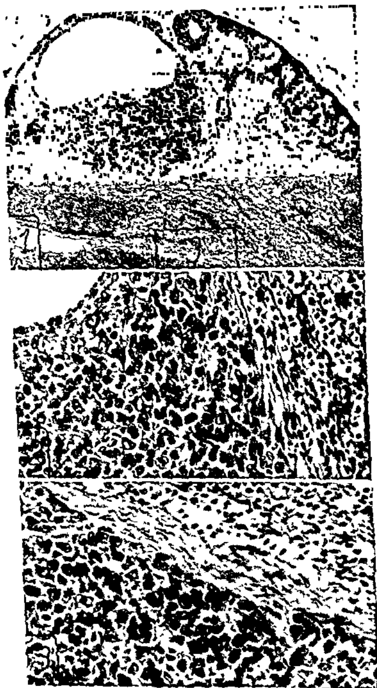
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BRIEF REPORT

HIGH INCIDENCE OF MALIGNANCIES IN PERSISTENT ESTROUS RATS

By *T Vanha Perttula* and *V K Hopsu*

It has been repeatedly demonstrated that a state of persistent cornification of vaginal epithelium can be induced in rats experimentally with various procedures Such a state can be produced by transplantation of testis in female rats after birth by injections of androgenic steroids soon after birth by parabiotic union with gonadectomized partners by hypothalamic lesions ligation of oviducts etc. (for ref see *Takewaki* 1962) The ovaries of the persistent estrous rats contain follicles of varying sizes but lack corpora lutea Many investigators have shown however that the follicles in these ovaries can respond both to endogenous and exogenous gonadotrophins by transforming them into corpora lutea No major changes have been reported in organs other than hypophyseal gland and the sex organs even during long observation periods

In this preliminary report a high incidence of highly metastasizing abdominal malignancies initiating most likely in ovaries in persistent estrous rats is reported A full description of the results of histological histochemical chemical and electron microscopic studies will be reported later

Seventy four offsprings of Long Evans rats were injected subcutaneously with 1 mg testosterone propionate (Neo Hombreol® Organon) and eighty three offsprings in the same way with testo
day after birth Thirty five
animals were kept in equal
of the animal keeper The vaginal cycle of the rats was followed during two weeks when the rats were 100 days old by examining the vaginal smears after Shorr staining All the injected rats were in a stage of persistent vaginal cornification while the controls showed a normal cycle

About twelve months later several animals became swollen in the bellies and appeared to be in week physical condition These clearly suffering animals were sacrificed and autopsied In autopsy the abdominal cavity of the animals appeared to be full of bloody liquid with free tumour cells as revealed in smear preparations The mesenterium was frequently full of soft easily breakable gray or in places red stippled tissue mass with numerous haemorrhages The growing mass was always connected with both ovaries and frequently with uterine tubes retroperitoneal space between the kidneys and around the mesothelial coverings near the liver and stomach In numerous cases voluminous metastases of similar tissue growth have been found in the mediastinal space as well as in the lungs with pleural exudate Up to this time when the animals are fifteen months old 36 injected rats (?3 per cent) have developed similar malignant tumours and new cases appear at few days intervals In one rat a solid white subcutaneous tumour has been found in the area of mammary glands Histologically it was a fibroadenoma No tumours have been found in the untreated control rats

Histology of the ovaries and of the tumour tissue as revealed in ordinary paraffin sections stained with routine hematoxylin and eosin method is seen in Figs 1 2 and 3 Fig 1 shows an ovary at low magnification there is one small and one enlarged highly hyperplastic f
tumour tissue is seen
follicle is seen in Fi
varying in size and sl
chromatin and with numerous mitotic figures On the right in the figure there is normal ovarial stroma The stroma was seen in some places to be infiltrated by the cells from the enlarged follicle Fig 3 presents a part of the extra ovarial tumour tissue fixed in the ovarial capsel The cells in this tumour as well as in all of the

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Department of Pathology II University of Uppsala Uppsala Sweden

RADIOAUTOGRAPHIC EXAMINATION OF NUCLEIC ACID AND PROTEIN LABELLING OF ADENOSINE-TREATED TISSUE CULTURE CELLS

By

INGRID STENRAM, HANS RYBERG and UNNE STENRAM

Received 18 xii 64

The nucleoli of tissue culture cells are normally rather homogeneous However, treatment with adenosine gives a filamentous appearance to the nucleoli (*Hughes* 1952, *Lettre & Siebs* 1955) The concomitant metabolic changes are not known It was therefore considered of interest to study the nucleic acid and protein labelling with the radioautographic method following administration of tritiated amino acids and cytidine

MATERIAL AND METHODS

Monkey (Cynomolgus) kidney cells were obtained as monolayers contained in tubes from the State Bacteriological Institute Stockholm Sweden The cells were trypsinized and transferred to slides fitted with glass rings as described elsewhere (*Bergman Stenram & Stenram* 1963) The cells were incubated in a medium consisting of Hank s solution with 5 per cent calf serum and 100 I U penicillin/ml and 100 γ streptomycin/ml at 37° C in moist air with 5 per cent CO_2 in a covered chamber The cells were then left for 48 hours during which time they adhered to the slides and flattened The medium was then replaced with the same medium containing 2

without adenosine After a further 2 hours at 37° C the media and the rings were removed from the slides The slides with the cultures were washed for a few seconds in physiological saline solution at room temperature fixed in Carnoy s fluid No II for 5 minutes and passed through two 3 minute changes of absolute alcohol to xylene which was changed three times The slides were stored in xylene for practical reasons prior to the preparation of radioautographs Then they were brought through alcohols to water and treate
min to dissolve acid soluble
redistilled water and dried
where (*Stenram* 1964) using

Two slides with monolayers were used for each labelled substance The whole experiment was performed twice with different sets of cells

Grain counts were performe
methionine using a pair of ×
bjective N A 1.30 50 random
The figures for 5 consecutive s
btained for each slide the mean the standard error and the standard deviation of the mean were calculated these are given in the Table

tissue samples taken from tumour masses look identical to those found in the hyperplastic follicle seen in Fig. 2. The cells are tightly packed and very minimal connective tissue components are found. In larger tumour masses hemorrhagic sites are frequently seen. These findings suggest that the origin of the tumour is in the hypertrophied follicles which have gone through a malignant transformation.

An effort was taken to transplant the tumour cells present in the peritoneal exsudate and those in the tumour by injecting the peritoneal exudate as well as tumour homogenate prepared in saline into the peritoneal cavity, gluteal muscles and subcutaneously in the back in normal female rats. Only slow growth of the tumour or usually no growth was found in normal female rats while a vigorous growth took place in ovariectomized rats during three weeks observation period.

The high incidence of tumours in the rats injected with androgen hormones perinatally with no tumours in equal control rats kept in identical laboratory conditions is to be taken to demonstrate that the tumour growth is related to the hormone injection and most likely to the persistent estrous state of the rats. The histological picture and the wild invasive and mestastasizing growth of the tumours described show that they are highly malignant. Preliminary experiments suggest that the malignancies are transplantable and hormone dependent. The site of origin of the tumours is most likely the follicular epithelium and consequently the tumour can be taken to belong to the group of granulosa cell tumours.

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INGRID STENRAM, HANS RYBERG and UNNE STENRAM

Received 18 xii 64

The nucleoli of tissue culture cells are normally rather homogeneous However, treatment with adenosine gives a filamentous appearance to the nucleoli (*Hughes* 1952, *Lettre & Siebs* 1955) The concomitant metabolic changes are not known It was therefore considered of interest to study the nucleic acid and protein labelling with the radioautographic method following administration of tritiated amino acids and cytidine

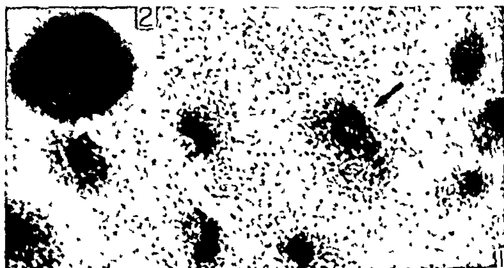
MATERIAL AND METHODS

Monkey (Cynomolgus) kidney cells were obtained as monolayers contained in tubes from the State Bacteriological Institute, Stockholm Sweden The cells were trypsinized and transferred to slides fitted with [illegible]

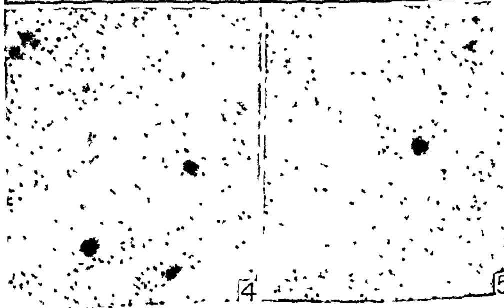
[illegible] the adenosine [illegible] [illegible] edium was [illegible] cytidine-³H [illegible] hionine-³H [illegible], England) [illegible] to the same manipulations but were given the media without adenosine After a further 2 hours at 37° C the media and the rings were removed from the slides The slides with the cultures were washed for a few seconds in physiological saline solution at room temperature, fixed in Carnoy's fluid [illegible] for 5 minutes and [illegible]

[illegible] and finally rinsed in three changes of redistilled water and dried The radioautographs were obtained as described elsewhere (*Stenram* 1964) using Ilford K2 fluid emulsion and an exposure of 14 days [illegible] belled substance The whole [illegible] f cells.

[illegible] which involved leucine and [illegible] s and a × 100 [illegible]


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OBSERVATIONS

General Morphology

The treated cells showed the filamentous appearance of the nucleoli described by previous authors. This was not so evident in the Carnoy fixed cells, employed for radioautographic purposes, as in cultures fixed according to *Serra* (cf. *Lettre & Siebs* 1955).

Experiment with Cytidine

The controls (Fig. 1) showed a heavy labelling over nucleoli, a somewhat weaker labelling over the non nucleolar part of the nucleus and still less labelling over the cytoplasm. In many cells, however, the heaviest labelling was over the nucleolar periphery with weaker labelling over the nucleoli (Figs. 1, arrow, and 2).

Adenosine (Fig. 3) largely suppressed the nucleic acid labelling in all parts of the cell. No grain counts were needed to prove this. The heaviest labelling remained in the nuclei.

Experiment with Leucine and Methionine

Both experimental and control cells appeared to attain similar or rather similar levels of labelling (Figs. 4 and 5). This impression was confirmed by grain counts in random square fields (Table 1).

TABLE 1

Number of Silver Grains in Square Fields

		Leucine		Methionine	
Exp. I	Control	304 ± 11	275 ± 17	175 ± 7	208 ± 10
		s = 35	s = 55	s = 21	s = 32
	Adenosine	331 ± 14	219 ± 13	182 ± 4	200 ± 8
		s = 43	s = 42	s = 14	s = 25
Exp. II	Control	346 ± 9	443 ± 12	255 + 6	252 ± 8
		s = 27	s = 37	s = 20	s = 24
	Adenosine	246 ± 8	213 + 19	155 ± 7	160 ± 4
		s = 27	s = 59	s = 21	s = 13

s is the standard deviation of the mean. For further explanation see section on Material and Methods.

Figs. 1-5

Radioautographs after administration of labelled cytidine (Figs. 1-3) and leucine (Figs. 4 and 5). × 1 000.

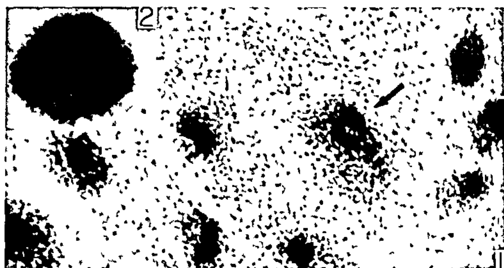
Fig. 1. Control. The labelling is as a rule heaviest over nucleoli, somewhat weaker over the non nucleolar part of the nucleus and still less intense over cytoplasm. The arrow points to a cell with heaviest labelling over the nucleolar periphery.

Fig. 2. Control. A cell with heavy labelling over the nucleolar periphery.

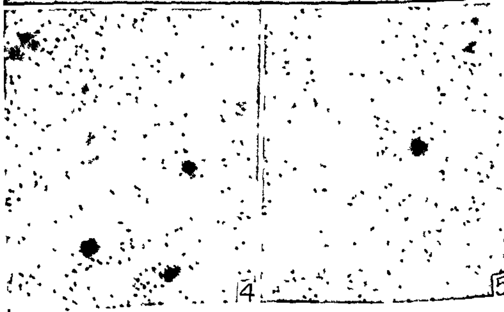
Fig. 3. Adenosine treatment. The labelling is much weaker all over the cell. An unlabelled cell in mitosis is seen to the right.

Fig. 4. Control. Labelling is seen over all the cell.

Fig. 5. Adenosine treatment. The labelling is similar to that in Fig. 4.


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Figs 1 5

Radioautographs after administration of labelled cytidine (Figs 1 3) and leucine

Fig 1 Control
over the weaker
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periphery

Fig 2

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DISCUSSION

The concentration of labelled nucleic acid at the nucleolar periphery (Figs 1, arrow, and 2) was first described in several tissues with large nucleoli in mice and rats, as the liver and pancreas, and also in heart muscle and kidney epithelium (*Stenram* 1962 a and b) *Monesi & Crippa* (1964) mentioned unpublished observations of this type in mouse liver cells by *Monesi* This labelling has also been seen in HeLa cells (*Feinendegen & Bond* 1963) and, by electron microscopic radioautography, in monkey kidney cells in culture (*Granboulan & Granboulan* 1964) These observations call attention to the importance of the nucleolus associated chromatin in nucleic acid synthesis, maybe primarily of messenger RNA (ribose nucleic acid)

Although normal optical microscopy of tissue culture cells treated with adenosine shows changes only in the nucleoli (cf *Hughes* 1952 *Lettre & Siebs* 1955), radioautography revealed an almost complete suppression of the nucleic acid labelling all over the cell (Figs 1-3) Protein synthesis was not or much less impaired The information necessary for this process thus remains in the cell to a considerable extent, although the synthesis of RNA, or at least RNA with a normal cytidine content, is almost completely stopped A parallel is found with actinomycin This substance, in concentrations of 1 3 ond 0 05 μg ml was added to other slides with cells of the same cultures RNA synthesis was largely suppressed, especially in the nucleoli and cytoplasm, but protein synthesis continued during the 2 5 hours of the experiment This is essentially in agreement with the results obtained by *Reich et al* (1962) with mouse fibroblasts strain L-929

Adenosine may be a suitable substance for further studies on the relationship between the nucleic acid and protein synthesis of various intracellular structures

SUMMARY

Tissue culture cells were treated with adenosine Cytidine-^{3}H, leucine-^{3}H and methionine-^{3}H were added Radioautography revealed an almost complete inhibition of nucleic acid labelling all over the cell Protein synthesis was not or much less impaired

This work was supported by a research grant from the *Swedish Medical Research Council* (project No W 337)

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Neurosurgical department, Rikshospitalet, Oslo (Head. professor dr med Tormod Hauge) and Institute of Pathological Anatomy (Head. professor dr med Olav Torgersen), Section of Neuropathology, Rikshospitalet, Oslo

MENINGIOMA WITH METASTASES TO CERVICAL LYMPH NODES

Case Report

By

ROALD OPSAHL JR and AAGOT CHRISTIE LØKEN

Received 24.xi.64

Extracranial metastases from intracranial tumours are rare incidences From a survey of the litterature 23 cases of verified metastases from meningiomas (*Abbot & Love* 1943, *Christensen et al* 1952, *Cross & Cooper* 1952, *Vlachos & Prose* 1958, *Kruse* 1960) have so far been reported, and as stressed by *Zulch* (9) new cases should still be reported



Fig 1
Section from the tumour that was removed in 1954
Haematoxylin eosin stain × 208



Fig 2

Photograph january 1963 Metastatic nodules can be seen lifting the skin in the neck Biopsy scar in the lower part

K R R a 70 years old male who had suffered *from a schizophrenic* psychosis *since 1927* was admitted to hospital in october 1954 because of an egg sized intumescence in his left fronto-temporal region It had *been* slowly growing since *1950-51* On examination he presented symptoms *of a paranoid* schizophrenia His *neurological* status was negative X ray of the skull showed irregular bone formation in the basal part of the tumour and *eroded bone* underlying it Carotid angio*graphy* and pneumoencephalography failed to *reveal any* intracranial expansion *Electroencephalography* showed slight hypersynchrony over the left temporal region

A biopsy showed a cellular tumour composed *of polygonal* or elongated cells *growing* in sheaths … stological diagnosis was

… Rikshospitalet in october
… r a small defect was found
… tissue that infiltrated
… brum Also the intra-
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… n various *areas of the*
… ers, the cytoplasm and
… short formation was frequent
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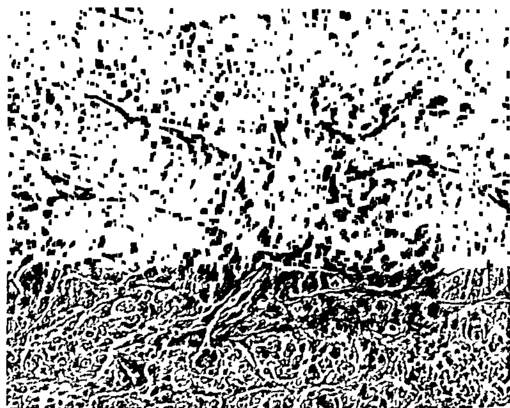


Fig 3
Section from a subcutaneous nodule in the neck
Haematoxylin eosin stain, × 208

(Fig 1) Histological diagnosis Cellular meningioma with some atypical signs Meningothelial type

6 years later, in december 1960 the patient was readmitted and this time 2 hard tumour nodules were present in his left fronto parietal region They measured 3 × 2 cm Neurological examination was again negative X-ray gave no conclusive evidence of any intracranial expansion He was not reoperated

In december 1962 he was again admitted The local recurrences had increased in size In addition several hard, indolent subcutaneous nodules were found on palpation behind the ear and in the lower cervical area on the left side They were up to walnut sized (Fig 2) One of the nodules was removed for histology It was found to be surrounded by a connective tissue capsule containing small daughter nodules The cells formed bands and whorls they were mostly elongated with indistinct borders There was some variation in the size of the nuclei, many of them contained coarse chromatin granules and mitoses were numerous Histological diagnosis Metastases from malignant meningioma (Fig 3) Otherwise the picture was very similar to that of the tumour removed 8 years previously A left sided subclavian angiography showed that both the local tumour recurrences and the tumours in the neck received their blood supply from the external carotid artery (Fig 4)

COMMENT

Most often when meningiomas have metastasized the secondary growths are found in the lungs, pleura, kidneys or bone marrow Only 2 similar cases are found described in the literature *Lima* (7) found a parasaggital meningioma that recurred after several attempts of



Fig 4
Left sided subclavian angiography Branches from the external carotid artery can be seen supplying the primary and metastatic tumours

removal It had *produced* daughter nodules around the scar in the scalp and a large mass in the cervical region with a histological picture identical with the primary tumour *Laymon & Becker* (6) reported a case where the meningioma had eroded the scull invaded the scalp and metastasized to the cervical lymph nodes In their case the histological picture described was similar to that in our case and according to *Cushing & Eisenhardt*'s classification a meningothelial type of meningioma

SUMMARY

Report is given of one case of a recidivating meningioma with metastases to the cervical lymph nodes on the same side



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Haematoxylin eosin stain, × 208

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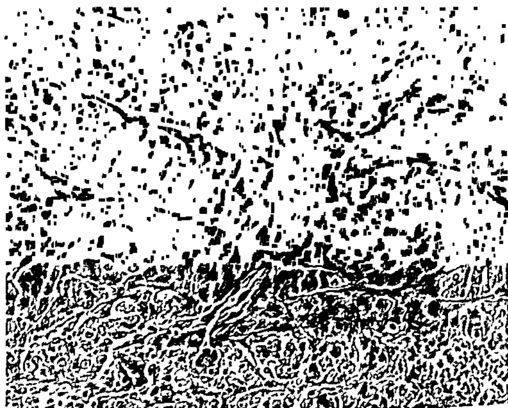


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PHENYLALANINE LEVELS IN BLOOD AND URINE IN NEWBORN INFANTS, MEASURED BY GUTHRIE TEST

By

I RNA LUND KLAUS VOLLMOND and BJARNI ØYLISEN

Received 6 i 65

As the introduction of the *Guthrie* test (*Guthrie* 1961 *Guthrie & Susi* 1963) for screening all newborn infants for phenylketonuria (P K U) is being considered in Denmark it is desirable to know whether the range of values for phenylalanine in serum from normal infants and phenylketonuric infants overlap during the first days of life

Brandon & Ashley (1963) found < 4 mg of phenylalanine per 100 ml using Guthrie s method both on 346 specimens of blood from newborn infants and on 170 specimens of urine from some of these children they found good correlation between the methods of *La Du* (*La Du & Michael 1960*) and Guthrie They recommended Guthrie s test for screening purposes in these words We think that the Guthrie inhibition assay method has sufficient merit to warrant nationwide participation in an attempt to eradicate phenylketonuria as a cause of mental retardation

Partington & Sinnott (1964) studied Guthrie s test on 6000 specimens of blood and found linear relation between the logarithm of the concentration of phenylalanine in the blood and the diameter of the bacterial growth zone on the Guthrie test The maximal sensitivity of the test was in the range from 1 to 10 mg of phenylalanine per 100 ml of blood it was less accurate at higher concentrations

Using this method clear cut differences were found between the blood from 66 patients with untreated phenylketonuria and that from 78 normal subjects (< 2 mg per 100 ml) The authors therefore recommended this test for screening as it is *simple comparatively cheap* and detects cases of phenylketonuria at an age when treatment is most effective No complications were met in the collection of blood from the newborn by heel prick These authors found *only one* false-positive Guthrie test in 2400 blood samples from newborn babies and none in a further 1734 from other sources This single "false positive" blood sample was from a premature baby giving rise to a growth zone some 2 mm in diameter but further Guthrie tests were normal

Hsia et al (1962) found moderately raised phenylalanine levels in the blood of premature infants in the neonatal period They fear that such babies may be regarded as cases of phenylketonuria and given unnecessary dietary treatment and therefore recommend that the blood level should be estimated by other methods, when the Guthrie test is found positive. *La Du's* method (*La Du & Michael* 1960) is more accurate than Guthrie's test, but is rather complicated and expensive, and about 10 ml of blood is necessary for one test *Guthrie*, (*Guthrie & Susi* 1963) recommends the use of paper chromatography for the confirmation of specimens found positive by his method

METHOD

Guthrie's "inhibition assay" has been used at Statens Seruminstiut for about two years On the whole the method is performed as described by *Guthrie* (*Guthrie & Susi* 1963) The control discs used contain the following concentrations

Blood 0 4 – 0 8 – 1 5 – 3 – 6 – 12 – 48 mg phenylalanine per 100 ml
Urine 1 5 – 3 – 6 – 12 – 24 48 – 96 mg phenylalanine per 100 ml

As Schleicher & Schuell No 903 filter paper used by Guthrie, was unobtainable different papers were compared and Whatmann No 31 E T chosen for further use

MATERIAL

Specimens from more than one hundred babies have been examined Blood and urine were absorbed onto filter paper (Whatmann No 31 E T) and mailed to the laboratory Blood from the newborn was taken by heelprick or from the ear

Blood

186 specimens from 122 newborn infants (aged from 0–60 days) were examined by the Guthrie test 145 specimens (= 78 per cent) were taken when the children were 0 7 days old, the largest number (44) being obtained on the second day (Table 1)

The average phenylalanine levels for each day were all = < 1 5 mg per 100 ml The level appears to increase from birth until the fourth day of life, but then remains constant at about 1 0 mg per 100 ml Examination of four specimens was impossible because of treatment with penicillin, blood and urine thus being inhibitory The average for the remaining (186 – 4 = 182) estaminations was 0 9 mg per cent phenylalanine

Only one child showed a blood level of 3 0 mg phenylalanine per 100 ml and another one of 2 0 mg per 100 ml the remaining 180 values were all < 2 mg per 100 ml (Table 2) The child having 3 0 mg per 100 ml was premature

Table 2 shows that 123 (67 6 per cent) specimens contained = < 1 0 mg phenylalanine per 100 ml, 54 (29 7 per cent) from 1 1–1 5 mg per

100 ml and only 5 (2 7 per cent) 1 6–3 0 mg per 100 ml Thus all 182 specimens of blood examined showed normal levels 3 mg per 100 ml or less the average being 0 9 mg per 100 ml

Premature Infants

[illegible] of the 122 infants examined were premature and 31 specimens [illegible] 1ᴐ1 [illegible] mg *per 100 ml* These levels are nearly the same, they are both within the normal range and show no evidence of raised levels in premature infants

TABLE 1

186 Specimens of Blood from 122 Infants Examined by Guthrie Test

Age in days	Number of specimens	Phenylalanine in mg per 100 ml	
		Range	Average
< 1	8	0 4 1 0	0 8
1	15	0 6 1 9	0 9
2	44	< 0 4 2 0	0 8
3	16	0 4 1 5	1 0
4	4	1 1 1 5	1 4
5	23	< 0 4 1 5	0 8
6	19	< 0 4 1 5	0 9
7	16	0 5 1 9	1 0
8	7	0 8 3 0	1 2
9	3	0 8 0 9	0 8
10	0		
11 15	7	0 8 1 5	1 0
16 20	4	0 4 1 0	0 6
21 30	9	0 4 1 3	0 9
31 40	2	0 8 1 5	1 2
41 50	0		
51 60	1	1 5	1 5
Unknown	4	0 4 1 5	0 9
Treated with penicillin	4		
In all	186	< 0 4 3 0	0 9

145 (78 0 per cent) specimens examined on 0– 7 days of life
17 (9 1) 8 15
13 (7 0) " 16 30 " "
3 (1 6) " " 31 60 " "
4 (2 2 " ") " " age unknown
4 (2 2) no results

TABLE 2

182 Specimens of Blood from 122 Infants Examined by the Guthrie Test

mg phenylalanine per 100 ml	< 0 4	0 4	0 5	0 6	0 7	0 8	0 9	1 0	< 0 4 1 0
Number of specimens	5	23	6	15	0	49	13	12	123 (67 6 per cent)

mg phenylalanine per 100 ml	1 1	1 2	1 3	1 4	1 5	1 1 1 5
Number of specimens	27	5	1	0	21	54 (29 7 per cent)

mg phenylalanine per 100 ml	1 6	1 7	1 8	1 9	2 0	3 0	1 6 3 0
Number of specimens	0	0	1	2	1	1	5 (2 7 per cent)

Hsia et al (1962) found moderately raised phenylalanine levels in the blood of premature infants in the neonatal period They fear that such babies may be regarded as cases of phenylketonuria and given unnecessary dietary treatment and therefore recommend that the blood level should be estimated by other methods, when the Guthrie test is found positive *La Du*'s method (*La Du & Michael* 1960) is more accurate than Guthrie's test, but is rather complicated and expensive, and about 10 ml of blood is necessary for one test *Guthrie*, (*Guthrie & Susi* 1963) recommends the use of paper chromatography for the confirmation of specimens found positive by his method

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100 ml and only 5 (2.7 per cent) 1.6–3.0 mg per 100 ml. Thus all 182 specimens of blood examined showed normal levels, 3 mg per 100 ml or less, the average being 0.9 mg per 100 ml.

Premature Infants.

29 of the 122 infants examined were premature and 31 specimens from these 29 babies gave an average level of 0.95 mg per 100 ml; 151 specimens from 93 infants born at term showed an average of 0.91 mg per 100 ml. These levels are nearly the same, they are both within the normal range and show no evidence of raised levels in premature infants.

TABLE 1

186 Specimens of Blood from 122 Infants, Examined by Guthrie Test

Age in days	Number of specimens	Phenylalanine in mg per 100 ml	
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<1	8	0.4 1.0	0.8
1	15	0.6 1.9	0.9
2	44	<0.4 2.0	0.8
3	16	0.4 1.5	1.0
4	4	1.1 1.5	1.4
5	23	<0.4–1.5	0.8
6	19	<0.4 1.5	0.9
7	16	0.5 1.9	1.0
8	7	0.8 3.0	1.2
9	3	0.8 0.9	0.8
10	0		
11 15	7	0.8 1.5	1.0
16–20	4	0.4 1.0	0.6
21 30	9	0.4–1.3	0.9
31 40	2	0.8–1.5	1.2
41 50	0		—
51–60	1	1.5	1.5
Unknown	4	0.4–1.5	0.9
Treated with penicillin	4		
In all	186	<0.4–3.0	0.9

145 (78.0 per cent) specimens examined on 0–7 days of life
17 (9.1 „ „) „ „ „ 8–15 „ „ „
13 (7.0 „ „) „ „ „ 16–30 „ „ „
3 (1.6 „ „) „ „ „ 31 60 „ „ „
4 (2.2 „ „) „ „ age unknown
4 (2.2 „) „ no results

TABLE 2

182 Specimens of Blood from 122 Infants, Examined by the Guthrie Test

mg phenylalanine per 100 ml	<0.4	0.4	0.5	0.6	0.7	0.8	0.9	1.0	<0.4–1.0
Number of specimens	5	23	6	15	0	49	13	12	123 (67.6 per cent)
mg phenylalanine per 100 ml	1.1	1.2	1.3	1.4	1.5	1.1–1.5			
Number of specimens	27	5	1	0	21	54 (29.7 per cent)			
mg phenylalanine per 100 ml	1.6	1.7	1.8	1.9	2.0	3.0	1.6–3.0		
Number of specimens	0	0	1	2	1	1	5 (2.7 per cent)		

Hsia et al (1962) found moderately raised phenylalanine levels in the blood of premature infants in the neonatal period They fear that such babies may be regarded as cases of phenylketonuria and given unnecessary dietary treatment and therefore recommend that the blood level should be estimated by other methods, when the Guthrie test is found positive *La Du's* method (*La Du & Michael* 1960) is more accurate than Guthrie's test, but is rather complicated and expensive, and about 10 ml of blood is necessary for one test *Guthrie*, (*Guthrie & Susi* 1963) recommends the use of paper chromatography for the confirmation of specimens found positive by his method

METHOD

Guthrie's inhibition assay' has been used at Statens Seruminstitut for about two years On the whole the method is performed as described by *Guthrie* (*Guthrie & Susi* 1963) The control discs used contain the following concentrations

Blood 0 4 – 0 8 – 1 5 – 3 6 – 12 – 48 mg phenylalanine per 100 ml
Urine 1 5 – 3 – 6 12 – 24 – 48 – 96 mg phenylalanine per 100 ml

As Schleicher & Schuell No 903 filter paper used by Guthrie, was unobtainable different papers were compared and Whatmann No 31 E T chosen for further use

MATERIAL

Specimens from more than one hundred babies have been examined Blood and urine were absorbed onto filter paper (Whatmann No 31 E T) and mailed to the laboratory Blood from the newborn was taken by heelprick or from the ear

Blood

186 specimens from 122 newborn infants (aged from 0–60 days) were examined by the Guthrie test 145 specimens (= 78 per cent) were taken when the children were 0–7 days old, the largest number (44) being obtained on the second day (Table 1)

The average phenylalanine levels for each day were all = < 1 5 mg per 100 ml The level appears to increase from birth until the fourth day of life, but then remains constant at about 1 0 mg per 100 ml Examination of four specimens was impossible because of treatment with penicillin, blood and urine thus being inhibitory The average for the remaining (186 -- 4 = 182) estaminations was 0 9 mg per cent phenylalanine

Only one child showed a blood level of 3 0 mg phenylalanine per 100 ml and another one of 2 0 mg per 100 ml the remaining 180 values were all < 2 mg per 100 ml (Table 2) The child having 3 0 mg per 100 ml was premature

Table 2 shows that 123 (67 6 per cent) specimens contained = < 1 0 mg phenylalanine per 100 ml, 54 (29 7 per cent) from 1 1–1 5 mg per

100 ml and only 5 (2 7 per cent) 1 6–3 0 mg per 100 ml Thus all 182 specimens of blood examined showed normal levels, 3 mg per 100 ml or less, the average being 0 9 mg per 100 ml

Premature Infants

29 of the 122 infants examined were premature and 31 specimens from these 29 babies gave an average level of 0 95 mg per 100 ml, 151 ... born at term showed an average of 0 91 mg

TABLE 1

186 Specimens of Blood from 122 Infants Examined by Guthrie Test

Age in days	Number of specimens	Phenylalanine in mg per 100 ml	
		Range	Average
< 1	8	0 4 1 0	0 8
1	15	0 6 1 9	0 9
2	44	< 0 4 2 0	0 8
3	16	0 4 1 5	1 0
4	4	1 1 1 5	1 4
5	23	< 0 4 1 5	0 8
6	19	< 0 4 1 5	0 9
7	16	0 5 1 9	1 0
8	7	0 8 3 0	1 2
9	3	0 8 0 9	0 8
10	0		
11 15	7	0 8 1 5	1 0
16 20	4	0 4 1 0	0 6
21 30	9	0 4 1 3	0 9
31 40	2	0 8 1 5	1 2
41 50	0		
51 60	1	1 5	1 5
Unknown	4	0 4 1 5	0 9
Treated with penicillin	4		
In all	186	< 0 4 3 0	0 9

145 (78 0 per cent) specimens examined on 0–7 days of life
17 (9 1) " 8 15
13 (7 0) 16 30
3 (1 6 " ") " " " 31 60 "
4 (2 2 ") " age unknown
4 (2 2) no results

TABLE 2

182 Specimens of Blood from 122 Infants Examined by the Guthrie Test

mg phenylalanine per 100 ml	< 0 4	0 4	0 5	0 6	0 7	0 8	0 9	1 0	< 0 4–1 0
Number of specimens	5	23	6	15	0	49	13	12	123 (67 6 per cent)
mg phenylalanine per 100 ml	1 1	1 2	1 3	1 4	1 5	1 1 1 5			
Number of specimens	27	5	1	0	21	54 (29 7 per cent)			
mg phenylalanine per 100 ml	1 6	1 7	1 8	1 9	2 0	3 0	1 6 3 0		
Number of specimens	0	0	1	2	1	1	5 (2 7 per cent)		

Comparison with other Methods

The Guthrie test on blood was compared with the *La Du* method (1960) A series of 15 determinations of the phenylalanine blood level in a two year-old PKU-boy treated with a diet was done simultaneously by both methods The results are given in Table 3 The average of the levels found with the Guthrie test was 7 7 mg per cent and with the La Du 7 1 mg per cent Thus these methods appear to give similar results Guthrie tests were done at Statens Seruminstitut and the La Du method at Medicinsk Laboratorium, Copenhagen (Dr *Moller*)

TABLE 3
Guthrie Test Compared with the La Du Method in 15 Determinations of Phenylalanine in Blood from a P K U Patient

Date	Phenylalanine in mg per 100 ml blood	
	Guthrie test	La Du method
25–9 63	28	24 5
14 10	30	32 2
21 10	10	10 5
28–10	2 0	2 1
4 11	1 5	2 3
11 11	3 0	0 7
18 11	3 0	2 2
25 11	3 0	2 6
2 12	2 0	1 8
9 12	1 5	2 1
16 12	8 4	10 1
21 12	9 0	5 9
28–12	7 5	4 4
3–1 64	3 0	2 6
10–1	3 8	2 9
Average	7 7	7 1

The Guthrie test on blood was also compared with chromatography (*E Lund & E Wamberg* 1962) Specimens of blood from 15 cases were examined by chromatography in Norway (Professor *Walaas*, University of Oslo) and by the Guthrie test in Denmark (Statens Seruminstitut) On an average the results by the Guthrie test were a little higher than those obtained by chromatography The best accordance was found in the lower values

Urine

147 specimens of urine from 102 newborn infants (Table 4) were examined by the Guthrie test 11 of these specimens could not be read 7 were contaminated with faeces and 4 inhibited the growth of the subtilis-strain

The smallest amount of phenylalanine in urine detectable by the Guthrie test is 1 5 mg per 100 ml 106 of the 136 specimens from normal

infants (78 per cent) gave no reaction and only 30 (22 per cent) contained from 1 5 6 mg per 100 ml

7 specimens gave results corresponding to > 10 mg per 100 ml, but in all of these the filter paper was obviously contaminated with faeces. Although no faeces could be seen on any specimen containing lower amounts, traces may nevertheless have been present, this is a disadvantage when urine for phenylalanine testing is collected from a diaper

TABLE 4

147 Specimens of Urine from 102 Infants Examined by Guthrie Test

	mg phenylalanine per 100 ml									Treated with penicillin
	< 1.5	1.5	2	3	4	5	6	~ 10	+ faeces 10	
Number of specimens	106	6	7	11	4	0	2	0	7	4

< 1 5 — 106 specimens of urine (= 78 per cent)
1 5 6 — 30 specimens of urine (= 22 per cent)
non readable — 7 + 4 = 11 specimens of urine
+ faeces = contaminated with faeces

In order to discover the phenylalanine content of *faeces* from newborn infants Guthrie tests were done on 27 specimens from non phenylketonuric children. 10 specimens of faeces from adults were also examined for comparison. For the reading of the test we used control discs with phenylalanine dissolved in urine. All specimens were examined twice and the average for each specimen is given in Table 5

TABLE 5

32 Specimens of Normal Faeces Examined by Guthrie Test

		Phenylalanine in mg per 100 ml faeces						Specimens in all
		5-9	10-14	15-19	20-24	25-29	30	
Number of specimens	infants < 18 months	6	12	2	2	0	0	22
	adults	3	1	4	1	1	0	10

All results are an average of two tests

Faeces from infants < 18 months contained up to 25 mg of phenylalanine *per 100 ml* and those from adults showed almost the same concentrations

4 of the 147 specimens of urine examined showed growth inhibition around the discs. In each case the children had been treated with *penicillin* some hours before the specimen was taken. It is impossible to measure phenylalanine level by Guthrie's test when the child is receiving penicillin

Another disadvantage of examining urine for phenylalanine is that the diuresis is not constant When a urine is diluted by a high diuresis the level of phenylalanine will be found lower than when the diuresis is smaller Thus there is no correlation between the concentration of phenylalanine in blood and in urine even when these specimens are taken at the same time

SUMMARY

186 specimens of blood from 122 infants and 147 specimens of urine from 102 of these were examined by Guthrie's inhibition assay and all showed normal content of phenylalanine There were no false positive reactions

29 premature infants showed the same blood level of phenylalanine as infants born at normal term

The Guthrie test was compared with the La Du method and showed very good agreement

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Acta path et microbiol scandinav 64, 305 308, 1965

The Virus Laboratory, Ullevål Hospital, Oslo Norway

SYNCHRONOUS DIVISION OF HAMSTER CELLS

By

GUDRUN BJELTVEDT[1]

Received 20 xii 64

During the past ten years a number of investigations of methods of induction of synchronous multiplication in mass cultures of bacteria, protozoa, algae, and cancer cells have been undertaken *Scherbaum* (1960) and *Maaloe* (1962) have published extensive reviews of the research work in this field

From the numerous methods which have been employed for the induction of synchronous division in an artificial way, temperature shocks have been selected for the present work Synchronous division of HeLa cells following a period of cooling is mentioned in a paper of *Gey et al* (1954) Otherwise synchronous division in cell cultures of mammalian origin only seems to have been reported by *Newton & Wildy* (1959), who used HeLa cells

The intention of the present work was to develop a method which would render a material of hamster cells homogeneous and accordingly fitted for transformation investigations

MATERIALS AND METHOD

Cells Hamster kidney cells line BHK21 clone C13, supplied by Dr *M Stoker* Institute of Virology Glasgow University were used

Growth medium The medium contained 10 per cent inactivated (56°) calf serum 0 45 per cent glucose, 0 2 per cent tryptose (Bacto Tryptose, Difco) 0 002 per cent phenol red in Eagles basal medium modified to contain twice the normal concentration of amino acids and vitamins, and 100 units of penicillin and 100 μg of streptomycin per ml

Conditions for synchronous multiplication Hamster cells in the logarithmic phase of growth were used The growth medium was removed and the cells were suspended with 0 05 per cent trypsin, 0 02 per cent Versene and 0 005 per cent glucose in phosphate buffered saline (*Dulbecco & Vogt* 1954) They were incubated at 37° C for 10 to 15 minutes, then centrifuged (1000 r p m), resuspended in the growth medium, counted in a haemocytometer, and diluted to a suitable concentration

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SUMMARY

186 specimens of blood from 122 infants and 147 specimens of urine *from 102 of these were examined by Guthrie's "inhibition-assay"* and all showed normal content of phenylalanine There were no "false positive" reactions.

29 premature infants showed the same blood level of phenylalanine as infants born at normal term

The Guthrie test was compared with the La Du method and showed very good agreement

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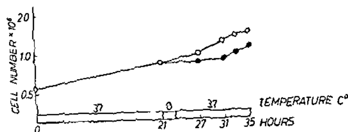


Fig 2

Growth of hamster cells at 37° C, during chilling at 0° C, and after the chilling period
○ Control cells, not chilled ● Cells chilled at 0° C for 2 hours

chilling conditions were tried: 7° C, 1 hour; 7° C, 3 hours, 15° C, 3 hours, 0° C, 2 hours; 0° C, 3 hours; and 0° C, 4 hours On chilling (the hamster cells) for 1 hour at 7° C, no multiplication in the cooling period was observed, but during the subsequent incubation at 37° C the cell growth ran parallel to the controls (Example in Fig 1). When the cells were chilled at 0° C for 2 hours the multiplication was inhibited during that period as well as in the following 7 to 11 hours Then the growth was parallel to the controls (Example in Fig 2) Cooling of the hamster cells at 0° C for 3 hours depressed their multiplication both during that period and for the following 9 to 13 hours, whereupon they increased 56 per cent to 79 per cent within 1 hour (Example in Fig 3). In a single experiment the cells were chilled at 0° C for 4 hours Their division was depressed for 14 hours after the chilling period, and then the cells increased 51 per cent during the following hour The experiments in Figs 1 to 3 are chosen as typical examples from 2 to 7 separate experiments The single points indicated in the figures were based on average values of 1 to 4 parallels

Control of cell growth Before the treatment with the trypsin-Versene mixture, the cells had adhered to the glass, and the medium could be removed carefully To counteract any further effect of trypsin-Versene

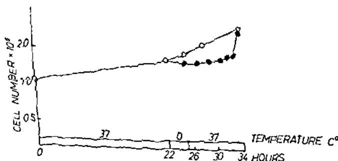


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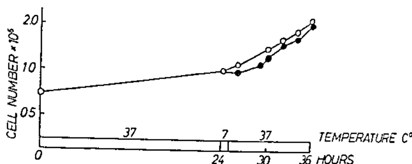


Fig 1

Growth of hamster cells at 37° C, during chilling at 7° C, and after the chilling period
○: Control cells, not chilled ●: Cells chilled at 7° C for 1 hour

The cell suspension was kept agitated by a stirrer during the dispensation, and 5 0 ml aliquots were dispensed into Jena G-20 bottles 10 0 ml of the growth medium were added per bottle The bottles were covered with tinfoil and then left horizontal in a humidified atmosphere of 5 per cent CO_2 at 37° C for the adjustment of pH in the growth medium Then the bottles were corked and placed in an incubator at 37° C When the cells in the course of approximately 24 hours were in good growth, they were cooled at 0° C for 3 hours and carried back to 37° C Cells which were not chilled served as controls

Control of cell growth The growth medium was sucked off carefully and replaced with 4 5 ml of the above-mentioned trypsin-Versene mixture, just covering the cells, and incubated at 37° C for 10 to 15 minutes Great care was taken to ensure that all cells had been detached from the glass This was controlled by microscopy With a pasteur pipette the cell suspension was quantitatively carried to a test tube containing 0 5 ml of calf serum Then every sample was counted in a haemocytometer Usually 2 to 3 samples were taken and 4 to 9 1 mm squares counted

RESULTS AND COMMENTS

Cells Stock of hamster cells used were kept in ampoules in fluid N_2 (—195° C) with 10 per cent dimethylsulphoxidum added Before use they were thawed and carried as culture for approximately seven days A shorter growth time would usually suffice, but in a few experiments hamster cells which had been carried as cultures for 10 to 11 days after thawing still had an edged and granulated appearance and showed poor growth Cells which had reached satisfactory growth were subcultivated every 4th or 5th day and taken from this when required

Conditions for synchronous multiplication The hamster cells usually increased 40 per cent to 50 per cent during the time chosen after dispensation, about 24 hours Variation of the cell number dispensed per bottle from $0 5 \times 10^6$ to $1 0 \times 10^6$ seemed to be unimportant The chilling temperature and the time of chilling were varied The following

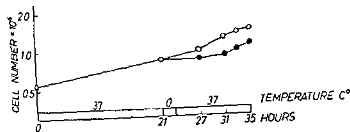


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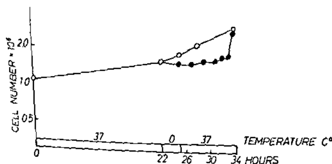


Fig 3

Growth of hamster cells at 37° C during chilling at 0° C, and after the chilling period
○ Control cells not chilled ● Cells chilled at 0° C for 3 hours

on the cells, 10 per cent calf serum was added to each sample The samples were counted in a haemocytometer at the time when the cells were dispensed, before the chilling, and at different times after chilling

SUMMARY

Synchronous division of hamster kidney cells, line BHK21, clone C13 has been observed after exposure to 0° C for 3 hours Cultures of hamster kidney cells were incubated at 37° C for approximately 24 hours after subculture, and then exposed to 0° C for 3 hours After replacement at 37° C samples were taken for cell counts The counting showed that only insignificant multiplication of the cells took place during the chilling period, and during the following 9 to 13 hours, whereupon they increased 56 per cent to 79 per cent within 1 hour

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ARTERIAL VASCULATURE OF THE MULTICYSTIC DYSPLASTIC KIDNEY

A Micro-Angiographical and Histological Study

By

ARNE LJUNGQVIST

Received 6 i 65

The term "renal dysplasia" has come into use during the last decade or so to denote a defective renal development, in which the kidney will be composed to a greater or lesser extent of primitive structures (*Baggenstoss* 1951) Such kidneys often contain cysts and in extreme cases they grossly seem to be composed mainly of cysts of varying size. For this type of dysplastic kidney the term of "congenital multicystic dysplasia of the kidney" (*Parkkulainen, Hjelt & Sirola* 1959), or "polycystic kidney type 2" (*Osathanondh & Potter* 1964) has been suggested

Detailed accounts of the histological appearance of the dysplastic kidney have been published (*Marshall* 1953, *Obiditsch-Mayer* 1956, *Ericsson & Ivemark* 1958, *Parkkulainen et al* 1959, *Potter* 1961), most of these accounts contain remarks on the apparently abnormal vasculature of the kidney However, the actual branching of the intrarenal arterial tree within the dysplastic tissue has not been investigated previously

The present parallel stereomicro-angiographical and histological examination of dysplastic kidneys, all of which were multicystic, was undertaken in an attempt to obtain new information about this type of congenital malformation and its histogenesis

MATERIAL AND METHODS

was bilateral

dneys micro

others were

histological

ations according to previously described procedures (*Ljungqvist* 1963a) Briefly these included filling the arterial system of the kidneys with a 7 5 per cent aqueous suspension of barium sulphate (Micropaque) which was injected through the main renal artery (or arteries) When the injection was completed the entire

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Fig 1

Micro angiogram of a frontal section of a multicystic dysplastic kidney showing the absence of cortico medullary differentiation Wide and winding arteries are seen to course from the hilar region towards the capsule The cysts are chiefly located sub capsularly and appear as rounded, avascular areas × 3 5

kidney was fixed in 10 per cent neutral formalin, embedded and sectioned in blocks 200–800 μ thick These were micro angiographed by a stereoscopic technique on a fine-grain photographic emulsion (Kodak Maximum Resolution Plate) The micro angiographed blocks were re-embedded and sectioned for histological examination One block from each of six kidneys was sectioned serially, from the other blocks three sections were taken The staining methods used were van Gieson's stain for connective tissue, Verhoeff's method for elastic fibres and haematoxylin eosin

TABLE

Case	1	2	3	4	5	6	7	8	9
Sex	F	F	M	M	F	M	M	M	M
Age	stillb	4 hrs	10 hrs	1 day	3 days	4 days	6 wks	12 wks	8 yrs

OBSERVATIONS

Macroscopical Appearance

The size of the kidneys varied from moderately larger than normal to severe hypoplasia In some kidneys the typical renal shape was largely preserved (Fig 1), while in others, particularly the hypoplastic



Fig 2

A Micro angiogram from a cyst wall Th ... ight
hich
thin

B ... seen
the
... with

... van Gieson × 35

ones, the shape was more or less distorted by protruding cysts However, all the kidneys contained numerous cysts, which varied in size from hardly discernible to that of a walnut The cysts were filled with a clear, watery material, and in no instance did a cyst contain contrast medium (F... ... ether, in ... ne central

Microscopical Appearance

Histology The cysts were lined with low cuboidal or flat epithelium, as a rule single-layered, and were surrounded by a dense, collagenous connective tissue (Fig 2 B) The intervening tissue was fibrous and contained nephrons, vessels and nerve trunks In some areas, particularly in the central portion of the kidney, the fibrous component predominated, in other areas nephrons were more numerous and



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OBSERVATIONS

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A B

Fig 2

A Micro angiogram from a cyst wall The avascular cystic space is seen to the right There is a pelvic vascular pattern of the cyst wall with spiralling arteries which course along its periphery and give rise to an anastomosing network of thin branches within the wall × 35

B ... all of the cyst is seen ... ification some of the ... These are filled with ... ast medium Van Gieson × 35

ones the shape was more or less distorted by protruding cysts However all the kidneys contained numerous cysts which varied in size from hardly discernible to that of a walnut The cysts were filled with a clear watery material and in no instance did a cyst contain contrast medium (Fig 1) In some regions the cysts were close together, in other regions there was an intervening tissue particlarly in the central portion of the kidney In no case was a pelvic cavity evident

Microscopical Appearance

Histology The cysts were lined with low cuboidal or flat epithelium, as a rule single layered and were surrounded by a dense, collagenous connective tissue (Fig 2 B) The intervening tissue was fibrous and contained nephrons vessels and nerve trunks In some areas, particularly in the central portion of the kidney the fibrous component predominated in other areas nephrons were more numerous and



A B

Fig 3

A Microangiogram of a malformed renal lobe showing an interlobar artery (center), which divides repeatedly forming arcuate and interlobular arteries (top). × 40

B Histological section of the area depicted in Fig 3 A A malformed lobe is seen which consists of an area of cortical tissue (above) and three converging primitive ducts van Gieson × 40

arranged as islands of cortical tissue with glomeruli and convoluted tubules (Fig 3 B) Particularly when dispersed singly in the fibrous tissue, the glomeruli displayed degenerative changes and some consisted of two or more separate tufts (Fig 4 B). Tubules emerged from the single glomeruli but, in the serial sections they were found to end blindly after a short course

No normal medullary pyramids were seen in any of the kidneys but tubules were occasionally found in juxtaposition to the areas of cortical tissue (Fig 3 B) These tubules generally had a narrow lumen (Fig 5 B), although occasionally they displayed slight cystic dilatation Their lumina were lined by stratified or single-layered epithelium of cuboidal or columnar cells Outside the epithelial lining there was a thick layer of concentrically arranged fibers of loose connective tissue, embryonic in type. Such tubules, which have been called "primitive ducts" (*Ericsson & Ivemark* 1958) were also encountered singly with no apparent positional relationship to areas of cortical tissue In three of the kidneys, including one of the cases of bilateral change (case 1), the loose connective tissue of the primitive ducts was abundant, and in many places



Fig 4

A Micro angiogram of an arteriole glomerular unit in the fibrous stroma The afferent arteriole is long and straight The glomerulus is divided into three tufts (top) × 75

B Histological section of the glomerulus depicted in Fig 4 A This consists of separate tufts and its proximal tubule tapers into the surrounding fibrous tissue van Gieson × 190

projected in a polypoid fashion into the lumen of the duct to give it a slit like appearance

All the kidneys were found to contain areas of small, dark cells, presumably representing erythropoiesis This occurred either as localized foci or as strands along the peripheral margin of the loose connective tissue of the primitive ducts The kidney of case 9 also displayed infiltration of inflammatory cells In some kidneys small islands of hyaline cartilage were encountered

Vascular morphology Like the histological appearance, the vascular patterns of the different kidneys were essentially similar in type and differed only in the degree to which the various abnormal components were represented The general pattern greatly deviated from the normal, mainly because of the lack of arrangement into cortical and medullary zones and to the presence of cysts (Fig 1) Thus, the arteries of the hilar region divided into large branches which branched repeatedly and followed a winding course towards the renal capsule The presence of numerous thick and winding arteries in the subcapsular area was a



Fig 5

A Micro angiogram of a primitive duct This courses across the length of the picture and is seen to be supplied by a bundle of thin and straight vessels These are derived from a common stem (bottom) Left and right a wide artery along the periphery of the duct × 75

B Histological section of the area depicted in Fig 5 A The lumen of the duct is slightly coiled and is seen in the section at irregular intervals It is lined by a single layer of tall and darkly stained epithelium and is surrounded by a thick layer of loose connective tissue embryonic in type The straight vessels depicted in Fig 5 A are seen within this connective tissue (t p) Left and right adjacent fibrous connective tissue van Gieson × 75

constant feature not seen in normal kidneys or in kidneys with secondary pathologic alterations (*Ljungqvist* 1963a) In the micro-angiograms the cysts were recognized as rounded avascular areas

The vasculature is presented in more detail in the following as an account of the vascular patterns characteristic of the various dysplastic tissue components

Cysts Highly spiralling arteries ran along the periphery of the fibrous walls of the cysts (Figs 2 A and B) These arteries were branches of the larger arteries that coursed from the hilar region towards the renal capsule and in turn they gave off smaller vessels into the fibrous cyst walls In these they gave rise to anastomosing networks of straight arterioles and capillaries

Primitive ducts Arteries were seen to curve along the periphery of the embryonic connective tissue of the primitive ducts Such arteries were branches of the hilar vascular tree and they gave off vessels into

the embryonic connective tissue These vessels were few in number, and after a short course they split up into bundles of thin arterioles, which followed a straight course parallel to the lumen of the duct (Figs 5 A and B)

Areas of cortical tissue Large arteries were regularly found to run along the border between an area of cortical tissue and the adjacent aggregation of primitive ducts (Figs 3 A and B) These arteries gave off branches into the cortical tissue, where arteriole glomerular units arose The glomeruli were generally small but otherwise apparently normal

The intervening tissue This fibrous tissue was fairly well supplied with wide and thick-walled arteries which followed a winding course At irregular intervals these vessels gave off arterioles which, after a straight and long course, formed glomerular tufts (Figs 4 A and B) These tufts were small with fibrotic foci and often divided into two or more tufts within the same capsular space In addition aglomerular arterioles were seen to arise from the wide and thick-walled arteries These arterioles divided repeatedly and finally formed capillary networks in the fibrous tissue In all but two kidneys (cases 5 and 7) the venous system of the intervening fibrous tissue was partially visualized It consisted of numerous sinusoidal vessels with abnormally thin, capillary like walls These vessels coursed close to the corresponding arteries The passage of contrast medium from the arterial to the venous side had probably taken place via the capillary network since no arterio venous anastomoses were demonstrated No particular vascular arrangement was recognized in relation to the foci of hyaline cartilage

DISCUSSION

The arterial vasculature of the abnormal structures in the multicystic dysplastic kidney has been demonstrated by the methods used in the present study

It was found that the fibrous walls of the cysts were supplied by networks of anastomosing arterioles and capillaries derived from highly spiralling arteries running along the peripheral border of the wall In the normal kidney such vascular arrangement is unique for the pelvic wall (*Hammersen & Staubesand* 1961, *Ljungqvist & Lagergren* 1962) The connective tissue of the primitive ducts, on the other hand, was supplied by bundles of thin and straight arterioles a vascular pattern which, in the normal kidney, is encountered only in the medulla The isolated areas of cortical tissue which were seen in most of the kidneys received branches from arteries that ran along the border between the cortical tissue and an adjacent aggregation of primitive ducts From their

judged as arcuate arteries The

then constitute the malformed

[illegible] of the cortical area in question The glomeruli dis

persed in the intervening fibrous tissue had long and straight afferent arterioles, a feature typical of the arteriole-glomerular units in the pelvic and periarcuate connective tissue of the foetal and infant kidney (*Ljungqvist* 1963b) Since these glomeruli do not normally degenerate and disappear until later in infancy, the occurrence of similar glomeruli, intact or degenerated, in the present material does not necessarily reflect a developmental abnormality However, the persistence of such glomeruli in the kidney of the 8-year-old girl (case 9) would seem to be abnormal, as would the occurrence of multicentric tufts in the other cases as well

In a recent microdissection study *Osathanondh & Potter* (1964) recognized four different types of polycystic kidneys Their type 2 corresponds to the multicystic dysplastic kidney of the present study In this type of malformation the authors observed that the ureter, on entering the kidney, divided into a system of tubules regarded as a tubular pelvis These tubules further divided into collecting ducts, which ended in the cysts after courses of varying length It was concluded that the cysts were the dilated ampullary ends of the collecting ducts and that the malformation of the kidney was due to an inhibition of the activity of the ampullae in inducing formation of nephrons in the renal blastema

In the present study, the only appreciable large tubules found were the primitive ducts, and it is evident that these correspond to the collecting ducts demonstrated by *Osathanondh & Potter* (1964) This is furher supported by the finding of a medullary vascular pattern in the connective tissue of the primitive ducts It is also obvious that the primitive ducts seen in the hilar region of the kidney correspond to the tubular pelvis of *Osathanondh & Potter* (1964), since these ducts were the only ones to be seen in the hilar region These ducts too, however, had a medullary type of vascular supply, the only structures with a pelvic vascular pattern were the cysts

If the concept of *Osathanondh & Potter* (1964) is applied to the findings of the present study, it appears that the ureteral branches within the kidney have a medullary vascular supply as long as they are tubular in structure and that they acquire a pelvic vascular pattern only when forming dilated cavities This points to the presence of a mechanism which makes the ureteral derivatives and the vascular system conform in types Whether, in the multicystic dysplastic kidney, a primary defect in the intrarenal ureteral branching has caused an abnormal development of the vascular system or the abnormal ureteral branching is secondary to a developmental abnormality in the vascular system cannot be ascertained However, the former explanation would seem the most plausible when recalling that some kidneys displayed an apparent overgrowth of the embryonic connective tissue of the primitive ducts with formation of polypoid invaginations of their lumina The vasculature of such ducts did not differ appreciably from that of other primitive ducts

SUMMARY

greatly from that of normal kidneys, mainly due to the abs[illegible] arrangement into cortical and medullary zones and to the presence of cysts

The vasculature of the cyst walls was similar to that of the normal renal pelvis The primitive ducts showed a medullary vascular pattern Since the cysts as well as the primitive ducts are known to derive from the ureter, the findings indicate that the intrarenal *ureteral branches* have a medullary vascular supply as long as they are tubular in structure and that they acquire a pelvic vasculature only when they form dilated cavities

Judging from their vascular patterns, areas of cortical tissue and adjacent aggregations of primitive ducts represent malformed renal lobes

Arteriole glomerular units with blindly ending tubules were encountered *in the fibrous stroma of the kidneys* On the basis of their appearance and localization these units could be considered to be the dysplastic counterparts to the arteriole glomerular units normally occurring in the pelvic and periarcuate connective tissue of the foetal and infant kidney

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SUMMARY

The arterial vasculature of ten multicystic dysplastic kidneys was studied by stereomicro-angiographical and histological techniques. The vascular patterns of these kidneys were essentially similar and deviated greatly from that of normal kidneys, mainly due to the absence of an arrangement into cortical and medullary zones and to the presence of cysts.

The vasculature of the cyst walls was similar to that of the normal renal pelvis. The primitive ducts showed a medullary vascular pattern. Since the cysts as well as the primitive ducts are known to derive from the ureter the findings indicate that the intrarenal ureteral branches have a medullary vascular supply as long as they are tubular in structure and that they acquire a pelvic vasculature only when they form dilated cavities.

Judging from their vascular patterns areas of cortical tissue and adjacent aggregations of primitive ducts represent malformed renal lobes.

Arteriole-glomerular units with blindly ending tubules were encountered in the fibrous stroma of the kidneys. On the basis of their appearance and localization these units could be considered to be the dysplastic counterparts to the arteriole-glomerular units normally occurring in the pelvic and periarcuate connective tissue of the foetal and infant kidney.

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Fibrinase (...) was obtained by the courtesy of Ass.-Prof. Inga Marie Nilsson, ... General Hospital, Malmö. According to the manufacturers it contained 3600 Ploug units ...

Stockh
et al
phosphate buffer a
containing 53.4 arb
to Wallén (1962)

Mayer (1961)

METHODS

serum showing 50 per cent hemolysis was considered to contain one 50 per cent hemolytic unit of C′ (CH_{50}).

Titration of components of complement was performed according to Pillemer et al. (1956).

Plasmin activity was measured on heated fibrin plates according to *Astrup & Müllertz* (1952) and *Lassen* (1953). The fibrinolytic activity was expressed as square millimeters of lysed area (obtained by multiplication of two perpendicular diameters of the lysis zone[1]).

activity was measured at pH 7.4, ionic strength 0.15, temperature +37° C, with a final ATPe concentration of 0.04 M and a total volume of 2.5 ml. Enzyme activity

being obtained with the same amount of enzyme by the present method. This is in

[1] Most of these determinations were generously performed by Dr. *L. Andersson*, Department of Urology, University Hospital, Lund, Sweden.

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INABILITY OF A HIGHLY PURIFIED STREPTOKINASE PREPARATION TO INACTIVATE COMPLEMENT IN SERUM

By

ANNA-BRITA LAURELL, B LUNDH and J MALMQUIST

Received 3 xii 64

Lepow et al (1954) found activation of human serum plasminogen by a streptokinase preparation (SK) to be accompanied by destruction of haemolytic complement (C′) activity C′4 was found to be the most sensitive component, C′2 and C′1 being affected less, and C′3 slightly or not at all It was concluded that the SK-activated plasmin was the factor responsible for C′ inactivation They also found that C′4 was destroyed only when C′1 was present in the reaction mixture

Their conclusion was compatible with the evidence presented by *Lepow et al* (1958), suggesting that upon SK activation the plasminogen present in crude C′1 preparations is capable of converting C 1 to C 1 esterase, which in turn is known to inactivate C′4 and C′2 (*Lepow et al* 1956a and b)

In the aforementioned studies the SK preparation used was Varidase®", (Lederle, U S A) It was observed in this laboratory that a more highly purified SK preparation ("Kabikinase®", AB Kabi, Stockholm, Sweden) failed to inactivate C′ This prompted us to investigate the plasmin and the C′ systems for any interrelationship The present paper is concerned with the effect of the following procedures on C′

1) incubation of fresh serum or plasma with the two preparations of SK,
2) incubation of serum with urokinase, which is known to activate plaminogen through a mechanism differing from that of SK activation (*Ploug & Kjeldgaard* 1957, *Kjeldgaard & Ploug* 1957),
3) incubation of serum with purified plasmin, and
4) infusion of Kabikinase into patients

MATERIAL

Fresh sera from 4 normal individuals
Citrated plasma from 2 normal individuals (1 vol of 3 8 per cent trisodium citrate plus 9 vols of blood, plasma separated at 1200 g for 20 minutes in the cold)

This investigation was supported by a grant from *Swedish Medical Research Council*

... ntration of 625, 1250 and 2500 units per ml, respectively, ... ne activity of C'1 esterase ... able 2)

Citrated plasma was incubated with the SK preparations in a final concentration of 667 units per ml Control samples were incubated with barbital buffer Aliquots were withdrawn after 5, 15, 30 and 60 minutes, ... bath and dialysed for 4 hours in the cold against two ... —60° C ... pidly and the fibrin clots were centrifuged down Table 3 gives the esults obtained in this experiment Here, too, a difference in C'-inactivating capacity was found between the two SK preparations, a difference that can hardly be ascribed to any difference in the extent of plasmin formation

TABLE 1
Effect of Incubation of Serum with Varidase or Kabikinase on C'4 C'2 and on Plasmin Activity

	Serum L O M treated with			Serum A B L treated with		
	Varidase	Kabikinase	Buffer	Varidase	Kabikinase	Buffer
C'4 (U/ml)	<50	600	600	<25	400	800
C'2 (U ml)	–			38	400	600
Plasmin activity (mm²)	18	18	0	12	8	0

	Serum J M treated with			Serum I T treated with		
	Varidase	Kabikinase	Buffer	Varidase	Kabikinase	Buffer
C'4 (U ml)	50	600	800	<25	100	800
C'2 (U ml)	75	400	600	–	–	
Plasmin activity (mm²)	26	13	0	12	26	0

TABLE 2
Effect of Incubation of Serum with Varidase or Kabikinase on C'1 Esterase Inhibitor Activity

Serum	A B L		J M	
Incubated at +3 C for 60 minutes with	C'1 esterase inhib (U ml)	Destroyed %	C'1 esterase inhib (U ml)	Destroyed %
Varidase final conc 2500 U ml	11	50	7	60
Kabikinase final conc 2500 U/ml	12	50	6	65
Varidase final conc 1250 U ml	14	40	7	60
Kabikinase final conc 1250 U ml	14	40	7	60
Varidase final conc 625 U ml	11	50	7	60
Kabikinase final conc 625 U ml	11	50	7	60
Buffer (control)	24		17	

accordance with the results obvious from the curves in the paper of *Pensky et al* (1961) The concentration of C 1 esterase inhibitor in serum samples was measured by adding 0 1 ml of the sample to the reaction vessel and then determining the decline in reaction velocity Control experiments showed that the addition of the same volume of buffer did not noticeably affect the reaction rate The unit of C'1 esterase inhibitor is defined according to Levy & Lepow (1959), one unit of inhibitor being the amount that neutralizes 10 units of esterase

Treatment of serum or plasma with streptokinase (SK) One volume of SK solution was incubated with two volumes of the sample at +37° C for varying periods The final concentration of SK in the mixture was, as a rule, 667 units per ml

Treatment of serum with urokinase 0 1 ml of UK solution was added to 0 5 ml of serum The mixture was then incubated at +37° C for 30 minutes The final concentration of UK varied from 100 to 1000 units per ml

Treatment of serum with plasmin Serum, undiluted or diluted 1 2 or 1 5 was incubated for 30 minutes at +37 C with an equal volume of the plasmin preparation

Administration of SK to patients The total dose infused ranged between 225 000 and 700,000 SK units (Table 6) The samples from the patients were assayed for fibrinolytic activity as described by *Nilsson & Olow* (1962) by determination[1] of

a) plasma euglobulin clot lysis time,
b) the activity of plasma on heated fibrin plates, and
c) fibrinogen

The administration of SK was followed by conversion of practically all the plasminogen to plasmin and almost complete disappearance of the plasma fibrinogen

RESULTS

1 *Treatment of Serum and Plasma, respectively, with Varidase or Kabikinase*

Incubation with Varidase in a final concentration of 667 units per ml serum for 60 minutes at +37° C caused a pronounced decrease in C'4 and C'2 in the sera investigated The same number of units of Kabikinase produced only a slight or no decrease of the titre In contrast, the plasmin activities of the samples were not found to differ significantly (Table 1) Even larger amounts of Kabikinase (up to a final concentration of 3330 SK units per ml) produced only a slight decrease of the C'4 titre In one of the sera incubation with Kabikinase caused a decrease in C 4 but less pronounced than after incubation with Varidase (Table 1)

The presence of SBTI in a final concentration of 1 mg per ml in the serum—Varidase mixture during incubation did not affect the destruction of C'4 The plasmin activity generated was not significantly lowered, however, and these results are therefore not conclusive Evidently SBTI did not inhibit plasmin when added to serum simultaneously with SK But when added to the serum after incubation of the latter with SK the same dose of SBTI completely inhibited the plasmin No attempt was made to find out the mechanism or implications of this finding

Treatment of serum or plasma with Varidase or Kabikinase in a

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Effect of Incubation of Serum with Varidase or Kabikinase on C'4, C'2 and on Plasmin Activity

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C'4 (U/ml)	<50	600	600	<25	400	800
C'2 (U/ml)	–			38	400	600
Plasmin activity (mm²)	18	18	0	12	8	0
	Serum J.M. treated with			**Serum I.T. treated with**		
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Effect of Incubation of Serum with Varidase or Kabikinase on C'1 Esterase Inhibitor Activity

Serum incubated at +37° C for 60 minutes with	A.B.L. C'1 esterase inhib. (U/ml)	A.B.L. Destroyed %	J.M. C'1 esterase inhib. (U/ml)	J.M. Destroyed %
Varidase final conc. 2500 U/ml	11	50	7	60
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C'2 (U ml)	75	400	600		–	
Plasmin activity (mm²)	26	13	0	12	26	0

TABLE 2

Effect of Incubation of Serum with Varidase or Kabikinase on C'1 Esterase Inhibitor Activity

Serum	A B L		J M	
Incubated at +3 C for 60 minutes with	C'1 esterase inhib (U ml)	Destroyed %	C'1 esterase inhib (U ml)	Destroyed %
Varidase final conc 2500 U ml	11	50	7	60
Kabikinase final conc 2500 U/ml	12	50	6	65
Varidase final conc 1250 U ml	14	40	7	60
Kabikinase final conc 1250 U ml	14	40	7	60
Varidase final conc 625 U ml	11	50	7	60
Kabikinase final conc 625 U ml	11	50	7	60
Buffer (control)	24		17	

TABLE 3

Effect of Incubation of Plasma with Varidase or Kabikinase for Varying Periods on C'1, C'4, C'2 and on Plasmin Activity

	Plasma A.B.L. incubated at +37 °C with								
	Varidase				Kabikinase				Buffer
Time (min.)	5	15	30	60	5	15	30	60	60
C'1 (U/ml)	200	100	100	100	600	400	400	300	400
C'4 (U/ml)	25	<25	<25	<25	600	300	300	200	400
C'2 (U/ml)	100	50	50	38	400	300	300	200	300
Plasmin activity (mm^2)	20	30	25	21	25	25	26	16	0

	Plasma J.M. incubated at +37 °C with								
	Varidase				Kabikinase				Buffer
Time (min.)	5	15	30	60	5	15	30	60	60
C'1 (U/ml)	400	100	75	50	800	400	600	400	600
C'4 (U/ml)	300	50	25	<25	800	600	600	600	600
C'2 (U/ml)	200	50	38	25	600	300	400	300	600
Plasmin activity (mm^2)	36	49	36	20	38	36	28	30	0

TABLE 4

Depression of Concentration of β_{1C} Globulin on Incubation of Serum with Varidase or Kabikinase

	Serum A.B.L.		Serum J.M.	
	Varidase	Kabikinase	Varidase	Kabikinase
Decrease of β_{1C} globulin %	40	25	50	25

Incubation of serum with Varidase or Kabikinase in a final concentration of 625, 1250 and 2500 units per ml, respectively, resulted in a decrease of the concentration of β_{1C}-globulin, which was most pronounced in the samples incubated with Varidase (Table 4). The decrease did not vary with the concentration of SK.

2. *Treatment of Serum with Urokinase*

Incubation of serum samples with the urokinase preparation resulted in high fibrinolytic activities. The titres of C' and of C'4 were unaffected (Table 5).

3. *Treatment of Serum with Plasmin*

After incubation of serum with plasmin to a final concentration of 26.7 ACU per ml a significant fibrinolytic activity was observed. As expected, fibrinolysis was most pronounced in the samples containing diluted serum, since normal serum contains potent plasmin inhibitors. The titres of C'4, C'2 and C'3 were not significantly lowered in the samples containing undiluted serum, but a slight decrease was found

serum diluted 1 ...
6) About 50 per cent of the C'1 esterase inhibitor was destroyed by this treatment

TABLE 5

Effect of Incubation with Urokinase at +37° C for 30 Minutes on C' C'4 and on Plasmin Activity in Serum

Serum with	C' (U/ml)	C'4 (U/ml)	Plasmin activity (mm²)
Urokinase 100 U/ml	59	600	61
Urokinase 200 U/ml	71	600	56
Urokinase 400 U/ml	71	600	54
Urokinase 1000 U/ml	59	600	40
No urokinase (control)	59	600	0

TABLE 6

Effect of Incubation of Serum with Purified Plasmin at +37° C for 30 Minutes on C'2 C'4 and C'3

	C'2 (U/ml)	C'4 (U/ml)	C'3 (U/ml)	Plasmin activity (mm²)
0 5 ml serum 1 1 + 0 5 ml plasmin	150	800	150	25
0 5 ml serum 1 1 + 0 5 ml buffer	200	1200	150	0
0 5 ml serum 1 2 + 0 5 ml plasmin	38	400	<25	59
0 5 ml serum 1 2 + 0 5 ml buffer	100	800	38	0
0 5 ml serum, 1 5 + 0 5 ml plasmin	<25	<25	<25	100
0 5 ml serum 1 5 + 0 5 ml buffer	38	200	<25	0

4 *Treatment of R1 with Varidase and Plasmin (respectively)*

No destruction of C'4 was found after incubation of R1 with Varidase, in accordance with the results of *Lepow et al* (1954)

Incubation of R1 preparations with plasmin in a final concentration of 26 7 ACU per ml at +37° C for 30 minutes was found to depress C'4 from 80 to <10 units per ml

5 *Sera from Patients Treated with Kabikinase*

The infusion of large doses of Kabikinase into the three patients investigated did not result in any significant change in C' or C'1 or C'4 titres despite the high plasmin activity noted *in vivo* (Table 7)

The concentration of β_1C globulin and the activity of C'1 esterase inhibitor were also determined No substantial changes in any of these components were observed, but the material is not large enough to warrant any conclusions about the effect of streptokinase on these two components *in vivo*

TABLE 3

Effect of Incubation of Plasma with Varidase or Kabikinase for Varying Periods on C'1, C'4, C'2 and on Plasmin Activity

	Plasma A B L incubated at +37° C with								
	Varidase				Kabikinase				Buffer
Time (min)	5	15	30	60	5	15	30	60	60
C'1 (U/ml)	200	100	100	100	600	400	400	300	400
C'4 (U/ml)	25	<25	<25	<25	600	300	300	200	400
C'2 (U/ml)	100	50	50	38	400	300	300	200	300
Plasmin activity (mm²)	20	30	25	21	25	25	26	16	0

	Plasma J M incubated at +37° C with								
	Varidase				Kabikinase				Buffer
Time (min)	5	15	30	60	5	15	30	60	60
C'1 (U/ml)	400	100	75	50	800	400	600	400	600
C'4 (U/ml)	300	50	25	<25	800	600	600	600	600
C'2 (U/ml)	200	50	38	25	600	300	400	300	600
Plasmin activity (mm²)	36	49	36	20	38	36	28	30	0

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Depression of Concentration of β_{1C} Globulin on Incubation of Serum with Varidase or Kabikinase

	Serum A B L		Serum J M	
	Varidase	Kabikinase	Varidase	Kabikinase
Decrease of β_{1C} globulin %	40	25	50	25

Incubation of serum with Varidase or Kabikinase in a final concentration of 625, 1250 and 2500 units per ml, respectively, resulted in a decrease of the concentration of β_{1C}-globulin, which was most pronounced in the samples incubated with Varidase (Table 4). The decrease did not vary with the concentration of SK.

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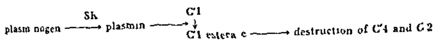
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DISCUSSION

The interaction between the plasmin and complement systems has been conceived as follows (*Lepow et al* 1954, *Lepow et al* 1958)



There is conclusive evidence that C 1 esterase can inactivate C 4 and C 2 (*Lepow et al* 1954, 1956a and b) Concerning the conversion of C 1 to C 1 esterase by SK the question arises whether this conversion and the resulting inactivation of C 4 and C 2 are mediated by activation of plasminogen to plasmin or by some other mechanism

The two SK preparations used had the same plasminogen activating effect but differed in capacity to inactivate C in human serum or [illegible] that the C inactivating effect of the SK prepara

latter assumption is strengthened by the finding that [illegible] activation by UK did not result in inactivation of whole C or C 4 in serum

As mentioned above incubation of diluted serum with purified plasmin resulted in destruction of a measurable amount of C 4 Similar results were obtained on incubation of an R1 preparation with plasmin indicating that the participation of C 1 esterase is not necessary for the destruction of C 4 in R1 This finding does not support the concept that the destruction of C 4 by plasmin is necessarily mediated by conversion of C 1 to C 1 esterase Since plasmin is a potent proteolytic enzyme and capable of digesting many serum proteins (*Ablondi & Hagan* 1960) it might be assumed that under suitable circumstances plasmin should be capable of inactivating components of C by simple proteolytic cleavage The absence of any effect of Varidase on C 4 in R1 and the inability of Kabikinase to destroy C 4 and C 2 in serum and plasma *in vitro* and *in vivo* also suggest that inactivation of C 4 in serum or plasma by Varidase is not caused by the simultaneous activation of plasminogen The amounts of plasmin generated in serum or plasma or R1 upon treatment with Varidase or equally well with Kabikinase are probably not sufficient to cause any significant direct destruction of C components The mechanisms involved in the destruction of C activity on addition of purified plasmin therefore appear to differ from those operating in the inactivation of C by Varidase

It is likely then that Varidase contains one or more components not occurring in Kabikinase and that these components in some way bring about destruction of C Concerning the mechanism involved at least two possibilities may be considered

1) The C inactivating factors act by destroying the C 1 esterase inhibitor with auto-activation of C 1 to C 1 esterase as a result

TABLE 7

Determination of Complement, Complement Components and of Fibrinolytic Activity in Samples from Patients Treated with Infusions of Kabikinase.

	I O			A K				H B		
	I	II	III	I	II	III	IV	I	II	III
Interval before (—) or after (+) infusion (hours)	−3	+2	+11	−24	+1	+4	+12	−24	+1	+1½
Total dose of Kabikinase (SK) infused (U × 1000)	0	250	700	0	100	225	625	0	150	225
C′ (U/ml)	154	154	83	154	154	135	111	154	154	154
C′1 (U/ml)	1200	600	400	—	—	—	—	—	—	—
C′4 (U/ml)	1200	600	600	1200	2400	2400	1600	2400	2400	1600
β_{1C}-globulin (mg/100 ml)	131	78	95	76	72	—	44	105	100	105
C′1 esterase inhibitor (U/ml)	29	—	21	15	16	9	16	16	13	12
Euglobulin clot lysis time (min.)	240	no clot	no clot	360	9	no clot	no clot	75	8	no clot
Citrated plasma on heated fibrin plates (mm^2)	0	177	277	0	81	100	218	0	305	381
Fibrinogen (g/100 ml plasma)	0.33	0.0	—	0.35	0.27	0.11	0.04	0.24	0.17	0.06
Total protein (g/100 ml serum)	6.2	4.8	4.2	7.6	6.8	—	6.6	7.6	7.1	6.1

— = not determined

2) Infusion of Kabikinase into patients with consequent plasminogen activation had no demonstrable effect on the serum C′ level

3) The two SK preparations did not differ in their capacity to inactivate C 1 esterase inhibitor. The effect on β_{1C}-globulin, on the other hand, was more marked after treatment of serum with Varidase than with Kabikinase

4) Activation of plasminogen in serum with UK, as well as treatment of undiluted serum with purified plasmin, did not result in C′ inactivation. Treatment of diluted serum, on the other hand, resulted in C′ inactivation presumably by proteolytic cleavage of the components

5) Plasmin inactivated C′4 in an R1 preparation while Varidase did not

It appears that inactivation of C′ by Varidase does not involve plasminogen activation, but is brought about by fixation of C′ to immune complexes between streptococcal antigens in Varidase and corresponding serum antibodies

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2) The C′ inactivating factors in Varidase are streptococc which differ from SK, and which react with antibodies normal sera with consequent fixation of C′

Lepow et al (1958) showed that treatment of serum with resulted in destruction of C′1 esterase inhibitor The mecha volved is unknown In the present study it was also shown th 50 per cent of the C′1 esterase inhibitor was destroyed on in of serum with purified plasmin in an amount comparable to that on incubation of serum with SK It may therefore be assumed destruction of C′1 esterase inhibitor on incubation of serum w is caused by the activated plasmin However, the two SK prepa used did not differ significantly in their capacity to destroy t esterase inhibitor, which seems to indicate that inhibitor destr is not related to C′ inactivation by Varidase

The inactivation of the individual components of C′ by SK (Vari is similar to that occurring during C′ fixation by antigen antibody plexes (*Pillemer et al* 1953, *Lepow et al* 1953). The inability of Var to inactivate C′4 and C′2 in an R1 preparation was interpreted by *L et al* (1953) as sign that Varidase exerts its effect by activation of p minogen to plasmin, and that plasmin inactivates complement sc by converting C′1 to C′1 esterase

An equally plausible explanation for the missing destruction of and C′2 in R1 on incubation with Varidase is that the C′ fixation antigen antibody complexes formed is not possible because R1 dose r contain C′1

The hypothesis of an antigen-antibody reaction leading to C′ i activation finds support in the fact that antibodies to a fairly larg number of streptococcal antigens occur in all normal sera (*Halbert et a* 1955) The finding of a certain degree of C′ inactivation by Kabikinas in one of the sera investigated here (Table 2) might be due to th serum containing antibodies to some antigen present also in this highly purified SK preparation, possibly to SK itself

Finally, as it is known that β_{1C} globulin is consumed in the process of complement fixation (*Muller-Eberhard & Nilsson* 1961), the greater decrease observed with Varidase is also consistent with the above com plement fixation hypothesis

SUMMARY

The effect of streptokinase (SK) on serum haemolytic complement (C′) was studied using two SK preparations of different purity Studies were also made with urokinase (UK) and purified plasmin The follow ing observations were made

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THE PASSAGE OF PARENTERAL AMPICILLIN INTO THE CEREBROSPINAL FLUID IN *HAEMOPHILUS INFLUENZAE* MENINGITIS

An Experimental Investigation in Rabbits

By

ARNE LITHANDER

Received 5 xii 64

The result of the treatment of bacterial meningitis is greatly dependent on the capability of the medicament in question to penetrate the blood cerebrospinal fluid barrier and thus reach the centre of infection We have already chemotherapeutic preparations to be used in cases of meningitis caused by *Haemophilus influenzae* bacteria, and now we have also a penicillin, namely ampicillin, with bactericidal effect on these micro organisms

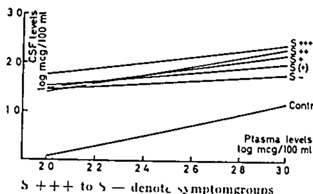
Relatively few *in vitro* investigations have been published concerning the sensitivity of *Haemophilus influenzae* bacteria to ampicillin, and in these tests the minimal inhibitory concentration (MIC) varied rather little *Robinson & Stevens* (1) found the MIC to range from 0 25 to 0 5 mcg *Knudsen et al* (2) from 0 1 to 0 5, *Stewart* (3) from 0 1 to 1 in 67 strains and *Klein et al* (4) from 0 2 to 1 6 mcg per ml in 52 strains

The experimental investigations of the passage of ampicillin through the blood-cerebrospinal barrier are also few in number and relatively sketchy In experiments on healthy rabbits *Acred et al* (5) found a concentration of 1 4 mcg per ml in the cerebrospinal fluid (CSF) one hour after intramuscular injection of 500 mg per kg body weight The concentration had decreased considerably only two hours after the injection *Auhagen et al* (6) could find no ampicillin in the CSF one hour after intravenous injection in a dosage of 5 8 mg per kg body weight

I therefore decided that it would be of interest to try to determine if ampicillin could penetrate the blood brain barrier in rabbits with *Haemophilus influenzae* meningitis

MATERIALS AND METHODS

The experiments were performed on adult rabbits weighing 2 to 2 5 kg The cisterna magna was punctured and 0 5 ml of CSF withdrawn Immediately thereafter each



S +++ to S — denote symptomgroups

Fig 1

CSF and plasma levels of ampicillin after intravenous injection of 12 000 mcg ampicillin per kg body weight in rabbits with *Haemophilus influenzae* meningitis Ampicillin concentrations expressed as log (mean number of mcg per ml)

detected between the concentration of ampicillin in plasma and the intensity of the signs of meningitis

The concentration of ampicillin in CSF (Table 1 and Fig 1) was consistently higher in rabbits with meningitis than in controls without meningitis In the experimental animals, the ampicillin concentration in CSF was formed to be different in the various symptom groups The concentration was much higher in group + + + than in the other groups There was a considerable concentration of ampicillin in CSF in rabbits with no clinical signs of meningitis (group —) The control rabbits showed only traces of ampicillin or none whatever in their CSF The covariance analysis revealed that the concentration of ampicillin in CSF rose significantly with the intensity of the signs of meningitis regardless of the concentrations of ampicillin in plasma

The concentration in CSF appeared to increase with the plasma concentration within each group The ratio $\frac{\text{ampicillin concentration in plasma}}{\text{ampicillin concentration in CSF}}$ $\left(\frac{\text{CSF}}{\text{P}}\right)$ for the different symptom groups is given in Table 1 It is also illustrated in Fig 1

The concentration of ampicillin in CSF and in plasma was closely related in all symptom groups The relationship was not significant, however, for the controls and symptom group — (correlation coefficient 0 335 and 0 41, respectively) The relationship was significant in symptom groups (+), +, + + and + + + (correlation coefficients, 0 75, 0 65, 0 81 and 0 85, respectively) With regard to the inclination of the regression line, the covariance analysis showed that the relation between the ampicillin concentration in CSF and in plasma was approximately the same in all symptom groups However, the level was much higher for all the experimental animals than for the controls Even before any clinical signs of meningitis became manifest in group —of the infected rabbits, the concentration of ampicillin in CSF was 14 per cent of that in plasma, *i e* ten times as high as in the controls In

roup (+) it was 19 per cent of the plasma concentration, and the level f the ratio further increasel in the remaining three symptom groups

There was no correlation between the concentration of ampicillin in CSF and the concentration of leucocytes and of *erythrocytes*

No ampicillin could be detected in the brain in any of the controls, in the rabbits in group — in most of the rabbits in groups + and + + or in three of the rabbits in group + + + In the remaining rabbits in group + + +, the concentration of ampicillin in the brain varied between 0 09 and 0 90 mcg per ml of brain substance, seemingly independent of the concentration in plasma

Microscopic examination revealed inflammatory changes in the meninges of the experimental animals The intensity of these changes corresponded on the whole with the intensity of the clinical signs of meningitis, *i e* group — There were no changes in the meninges in the controls

DISCUSSION

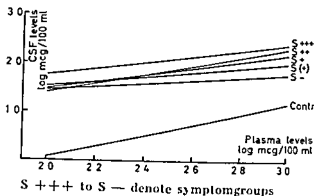
The findings hitherto obtained in investigations in man reveal a similarity between ampicillin and older forms of penicillin with regard to the passage through the blood CSF barrier Thus, the concentration of ampicillin in CSF in healthy subjects had been low, even following large doses

In experiments with intravenous or intramuscular administration of ampicillin in healthy animals, enormous doses were required before passage into CSF was achieved

In the present investigations in rabbits, the level of passage of intravenously administered ampicillin into CSF was relatively high in animals infected with *Haemophilus influenzae* bacteria by intracisternal injection The concentration in CSF was as high as or higher than required according to reports in the literature (1, 2, 3, 4), in order to check the growth of *Haemophilus influenzae* bacteria in vitro Also the increase of the CSF concentration was related to the plasma concentration

The passage of ampicillin through the blood CSF barrier was very poor in control animals without meningitis The relation between CSF concentration and plasma concentration in these animals was approximately the same as found earlier with benzyl penicillin in healthy control animals (7)

It is particularly noteworthy that there was a great difference between group — and the controls Any clinical signs of meningitis were not seen in these two groups Nevertheless there was a considerable difference between the two groups as regards the ampicillin concentration in CSF In group — the ampicillin concentration was on an average 0 4 mcg per ml in CSF in the controls only 0 05 mcg per ml In addition, in group the ampicillin concentration was high as related to the concentration in plasma which suggests that the permeability of the



S +++ to S — denote symptomgroups

Fig 1

CSF and plasma levels of ampicillin after intravenous injection of 12 000 ampicillin per kg body weight in rabbits with *Haemophilus influenzae* menin Ampicillin concentrations expressed as log (mean number of mcg per ml)

detected between the concentration of ampicillin in plasma and intensity of the signs of meningitis

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(Head Arne Lithander, M D)

AMPICILLIN THERAPY IN EXPERIMENTAL *HAEMOPHILUS INFLUENZAE* MENINGITIS IN THE RABBIT

By

ARNE LITHANDER

Received 5 xii 64

The present report concerns experiments with ampicillin therapy in

our arsenal

The literature contains no reports on ampicillin treatment of *Haemophilus influenzae* meningitis in man Nor does it contain any experimental studies on the subject In experiments in healthy rabbit *Auhagen et al* (1) found that ampicillin did not pass into the cerebrospinal fluid (CSF) following intravenous injection of small doses of ampicillin *Acred et al* (2) noted a relatively low concentration of ampicillin in CSF following very large intravenous doses In an earlier work *Lithander* (3) reported various workers observations of a minimal inhibitory concentration (MIC) of ampicillin for *Haemophilus influenzae* bacteria in tests *in vitro* which varied in most cases between 0 1 and 0 5 mcg per ml

MATERIALS AND METHODS

The experiments were made on rabbits weighing between 2 and 3 kg The animals were given injections in the cisterna magna of live *Haemophilus influenzae* bacteria which had been washed in physiological saline solution The bacteria had recently been isolated from clinical cases Between one and eight billion bacteria suspended in 0 5 ml of physiological saline solution were injected into the cisterna after the removal of 0 5 ml of CSF The sensitivity to ampicillin differed in the six different bacterial strains used The MIC varied between 0 05 and 0 5 mcg per ml The types of bacterial *strains used could not be determined*

The rabbits showed clinical signs of meningitis a day or two after the intra cisternal injection of bacteria On the bases of signs of meningitis they were divided

half

other

This

The

animals in each symptom group were assigned to three series by drawing lots Within each symptom group the rabbits in the first series were given intravenous

blood-CSF barrier was altered even before clinical signs of meningitis had appeared Here we find correspondence with the results with benzyl penicillin in rabbits infected with β-streptococci in the cisterna magna (8)

The microscopic examinations revealed correspondence between the ampicillin concentration in CSF and the pathological changes in the meninges Thus the increased permeability of the blood CSF barrier which could be observed even before the appearance of clinical signs of meningitis must have occurred because the pathological changes in the meninges preceded the clinical signs of meningitis

SUMMARY

1 Meningitis was produced in rabbits by the intracisternal administration of *Haemophilus influenzae* bacteria

2 Following intravenous administration, concentrations of ampicillin in CSF were as high as, or higher, than those which inhibited the growth of *Haemophilus influenzae* bacteria *in vitro* The concentration increased considerably in comparison with the CSF concentration in control rabbits even before clinical signs of meningitis could be noted

3 The concentration of ampicillin in CSF was related to the concurrent concentration in plasma and to the intensity of the signs of meningitis

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The signs of meningitis increased in intensity in rabbits which died during the observation period without having had diarrhoe This was true both of controls and of animals given ampicillin The length of survival after the beginning of the experiments varied greatly Most of the controls died within three to five days and most of the treated animals after seven to nine days

The results of the therapeutic experiments are shown in Table 1

TABLE 1

Results of Treatment with Intravenous Injections of Ampicillin in Rabbits with Haemophilus influenzae Meningitis

Symptom group at the first dose of ampicillin	48 000 mcg per kg		12 000 mcg per kg		Controls	
	N	% dead	N	% dead	N	% dead
(+)	24	8 3	17	11 8	35	22 9
+ and ++	22	18 2	10	49 0	27	70 4
Total	46	13 4	27	22 2	62	43 5

Dosage 48 000 and 12 000 mcg per kg body weight respectively

The table reveals that the mortality was lower for treated animals than for controls The difference was greatest when the signs of meningitis were distinct at the outset (symptom groups + and ++) The dose of 48 000 mcg per kg gave better results than 12 000 mcg per kg This difference was also greatest when the meningitis was pronounced at the outset When the signs of meningitis were dubious at the outset there was little difference between the rabbits given the 48,000 and the

vestigated only in 19
...y cisternal puncture

The interval between the last ampicillin injection and the cisternal puncture varied from 0 5 to 4 hours The concentration of ampicillin in CSF varied between 0 28 and 36 5 mcg per ml regardless of the interval after the last intravenous ampicillin injection

In the cases in which a sufficient quantity of CSF could be secured, the same specimen of CSF was used for the investigation on the presence of *Haemophilus influenzae* bacteria as for the determination of the ampicillin concentration In some rabbits the investigation was made after death in others at the end of the observation period Bacteria could be found in CSF from controls in 20 of the 22 cases examined The quantity of bacteria was moderate or large in most of the cases Two of the 32 cases treated with ampicillin showed occasional bacteria

At the beginning of the ampicillin treatment ten rabbits (not included in Table 1) had pronounced signs of meningitis and were practically moribund In these animals time allowed only four or five injections of ampicillin to be given before death occurred Though they had received

injections of 48 000 mcg of ampicillin[3] per kg body weight four times daily while those in the second series were given 12 000 mcg of ampicillin per kg body weight four times daily The rabbits in the third series served as controls and were given no treatment The smaller dosage was chosen since it had been used in a previous study on the passage of ampicillin through the blood brain barrier in rabbits with experimentally produced *Haemophilus influenzae* meningitis The rabbits were under observation for ten days after the bacterial injection into the cisterna The injections of ampicillin were continued until the end of the observation period *i e* eight or nine days Changes in the symptom picture were noted daily throughout the observation period The cisterna was punctured and samples of CSF were removed in some of the rabbits who died during observation and in some of the survivors The samples of CSF were used to determine the concentration of ampicillin and also for cultures to reveal *Haemophilus influenzae* bacteria The cultures were made on blood agar plates streaked with staphylococcus aureus Penicillinase was added to the samples in order to eliminate any growth inhibiting effect of the ampicillin The cisternal punctures were done at different intervals after the last ampicillin injection Immediately after
centration of ampicilli
method described in an
which were dosed with
first injection of ampici
per kg had the same symptom after the same interval Most of the rabbits with diarrhoea set in Rabbits with diarrhoea were not included in the tabulation of results Among the 65 rabbits dosed with 48 000 mcg per kg 19 (29 2 per cent) had diarrhoea Eighteen of these died Six out of 33 animals (18 2 per cent) given the dosage of 12,000 mcg per kg had diarrhoea Five of these died Diarrhoea was not observed in any of the controls Cultures were made of the faeces from some of the rabbits with diarrhoea bearing in mind the possibility of an invasion of staphylococci caused by the ampicillin treatment No staphylococci were found in any of the faecal tests only *E coli*

RESULTS

During the ten-day observation period, the clinical symptom picture in the animals which did not contract diarrhoea developed in varying ways In rabbits which survived there was a great difference between controls and animals which were given ampicillin In rabbits which belonged to symptom group (+) when the ampicillin injections were started, the signs of meningitis usually remained for only three to six days thereafter, sometimes following a brief increase in intensity The signs disappeared earlier in rabbits given the 48,000 mcg per kg dosage They were still present at the end of the observation period in approximately half the animals which had belonged to the symptom groups + and ++ when the treatment was started This was true regardless of the magnitude of the dose of ampicillin However, the signs had decreased in most of the animals given 48,000 mcg per kg and in a few of those given 12,000 mcg

The signs of meningitis were still present at the end of the observation period in most of the control rabbits which survived The intensity of the signs was usually unchanged but had increased in some cases This applied regardless of the symptom group to which the animal had belonged at the beginning of the observation period

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1 The ampicillin used in this investigation was kindly supplied by AB Astra Sodertalje Sweden The proprietary name of ampicillin from Astra is "Doctacillin"

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The Influenza Department Statens Seruminstitut Copenhagen Denmark

THE INFLUENCE OF STEROID HORMONES AND GROWTH HORMONES ON THE PRODUCTION OF INFLUENZA VIRUS AND INTERFERON IN TISSUE CULTURE

1 *The Influence of Hydrocortisone*

By

V Reinicke

Received 18 xii 64

Experiments by *Kilbourne et al* (7) and previous findings in this laboratory (12) have shown that the production of interferon in embryonated eggs infected with influenza virus decreased markedly when the eggs were inoculated with certain steroid hormones

Studies on the influence of two different steroid hormones on the production of interferon in tissue culture have been performed by *De Maeyer & De Maeyer* employing a continuous line of rat cells infected with Sindbis virus These experiments showed that steroids were able to reduce the production of interferon also in a tissue culture system (10)

The present experiments were undertaken to investigate the influence of hydrocortisone on the production of influenza virus and interferon in tissue cultures of chick embryo fibroblast This host cell system is less complex than the embryonated egg but employs cells derived from the same species, *i e* the chick embryo

MATERIALS AND METHODS

Most materials and methods have been described in detail in a previous paper (13)

i

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Sindbis virus stocks containing approximately 5 × 10[8] plaque forming units (p f u) per ml were prepared and stored as previously described The Sindbis virus was employed as challenge virus in titrations of interferon

Titration of virus Infectivity (FID50) and haemagglutinin titrations were carried out as previously described (12)

Production of interferon Interferon was produced in chick embryo fibroblast (c e f)

The author wishes to thank Mrs *B Saugbjerg* for skilled technical assistance

only very few injections of ampicillin *Haemophilus influenzae* bacteria could be found in CSF from only one of them

DISCUSSION

In the present therapeutic experiments with intravenous injections of ampicillin in experimental *Haemophilus influenzae* meningitis in the rabbit, the mortality was considerably higher in the untreated than in the treated rabbits The total mortality was distinctly lower in cases dosed with 48,000 mcg per kg than with 12,000 mcg This suggests that the concentration om ampicillin in CSF must be high if a therapeutic effect is to be achieved Evidence that this is the case is found in the fact that the signs of meningitis began to regress sooner when the dosage was 48,000 mcg per kg In most of the cases in which it was investigated, the ampicillin concentration in CSF was considerably higher than MIC for the meningitis-producing *Haemophilus influenzae* bacteria The fact that the CSF from a number of the treated rabbits was free from bacteria after only four to five injections indicates that the effect was rapid

SUMMARY

1 Meningitis was produced in rabbits by injection into the cisterna magna of *Haemophilus influenzae* bacteria

2 Four daily intravenous injections of ampicillin resulted in a mortality which was lower than the mortality in the untreated controls The difference was greatest between treated rabbits and controls when the signs of meningitis were distinct at the beginning of the treatment The mortality was lower in the treated rabbits when the ampicillin dose was 48,000 mcg per kg than when it was 12,000 mcg per kg

3 No *Haemophilus influenzae* bacteria could be detected in CSF in the majority of rabbits treated with ampicillin The bacteria had disappeared already after a few ampicillin injections in most of the rabbits which died soon after the beginning of the treatment

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MATERIALS AND METHODS

Most materials and methods have been described in detail in a previous paper (13)

Virus Influenza A WS in its 6–8 egg passage in this laboratory was employed for *production of influenza virus and interferon in tissue culture* This strain was selected because it is known to be a good inducer of interferon production (4, 17) Virus stocks containing $10^{9.2}$–$10^{9.7}$ LID50 per ml were prepared and stored as previously described

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tissue cultures grown in plastic Petri dishes having a diameter of 8 5 cm Following removal of the medium the cultures were inoculated with approximately $10^{6.5}$ EID_{50} of influenza A WS virus i e infection at a multiplicity of 0 1 0 3 After one hour of virus adsorption at 36° C the inoculum was removed the cultures were washed with 10 ml of Earle s BSS and 10 ml of maintenance medium were added to each culture After continued incubation at 36° C for 24 48 hours the medium was collected in activated and subsequently tested for interferon At the time of harvest no cytopathogenic effect could be observed in activation was carried out as described for half an hour As a control of the titrations were carried out

Titration of interferon Interferon was titrated by two methods

1) by a plaque inhibition technique as previously described (13) where two or four fold dilutions of interferon were added in 0 25 ml amounts to monolayers of c e f grown in plastic Petri dishes with a diameter of 8 5 cm Two to six cultures were used per dilution Control cultures received Earle s BSS Following incubation for one hour at 36° C approximately 60 p f u of Sindbis virus were inoculated into each culture and after continued incubation for one hour the cultures were overlayed with 5 ml of agar containing medium.

2) by a modification of method 1 two or four fold dilutions of interferon were added in one ml amounts to c e f monolayers grown in plastic Petri dishes 4 5 cm in diameter Two to six cultures were used per dilution Control cultures received Earle s BSS Following 3 hours incubation at 36° C the dilutions of interferon were withdrawn and approximately 60 p f u of Sindbis virus were inoculated into each culture After continued incubation for one hour the inoculum was removed and the cultures overlayed with 2 5 ml of agar containing medium

In both types of assays plaques were read after 40–48 hours incubation and the titre of interferon was expressed as the dilution of interferon which reduced the number of plaques to 50 per cent of that of the control cultures

Comparative titrations of batches of interferon showed that equal titres were obtained in the two assay systems though a tendency towards a slightly higher titre (less than two fold increase) was seen when assay 2) was employed (14)

Hydrocortisone A highly water soluble compound di (17 hydroxycorticosterone 21)phosphoriacidester sodiumsalt was employed dissolved in maintenance medium (13)

EXPERIMENTAL

An experiment originally designed to investigate the influence of hydrocortisone on viral interference (16) led to the observation that interferon was formed during influenza A WS virus infection in c e f cultures and also that hydrocortisone seemed to have an inhibitory effect on the production of interferon The experiment was performed as follows Duplicate c e f monolayers grown in 8 5 cm Petri dishes were preincubated for 24 hours with hydrocortisone 50 γ/ml Duplicate non treated cultures served as controls After removal of media the cells were washed with 10 ml of Earle s BSS per culture and subsequently each culture was inoculated with influenza A WS virus at a multiplicity of 0 1 0 3 After virus adsorption for one hour at 36° C the inoculum was removed, the cultures washed with 10 ml of Earle s BSS and replenished with maintenance medium After 24 hours of incubation at 36° C the fluids were harvested and pooled according to groups Media harvested from uninoculated cultures served as controls The pools of media were inactivated and subsequently tested for interferon No attempts were made to remove hydrocortisone from the medium before testing for interferon since previous experiments had

shown that hormones were without influence on the effect of preformed interferon when assayed in the present tissue culture system (*13*) It was found that a small amount of interferon appeared in the control cultures (the undiluted tissue culture fluid caused a 50 per cent reduction in number of plaques) while *no* interferon could be detected in the hydrocortisone treated cultures

In order to determine the content of interferon *both in media* and cells a similar experiment was performed Two concentrations of hydrocortisone (10 γ ml and 50 γ ml) were used, and media and cells were harve [illegible] 24 h [illegible] ncubation The media were assayed for interferon [illegible] ner [illegible] then scraped down in 2 5 ml of maintenance medium [illegible] orous pipetting the cells were frozen and thawed quickly three times The cellular debris was removed by light centrifugation and the supernatant was inactivated and subsequently tested for interferon A batch of *interferon* consisting of heat inactivated tissue culture fluid was included in the experiment as a reference This batch was also frozen and thawed quickly three times after which it was *inactivated* and subsequently titrated to ascertain that this treatment did not reduce the titre of interferon

In accordance with the preceding experiment a small amount of interferon (the undiluted tissue culture fluid caused a 50 per cent reduction in number of plaques) was detected in medium from influenza A WS inoculated cultures which had not been pretreated with hydrocortisone *The medium from cultures pretreated* with 10 γ ml of hydrocortisone also contained a small amount of interferon while no interferon could be detected in medium from cultures pretreated with 50 γ ml of hydrocortisone Interferon could not be detected in any of the supernatants from cell debris

The experiment shows that the low content of interferon in the hydro cortisone treated culture is due to a decreased production of interferon and not caused by a change in the permeability *of the cell membrane* tending to withhold the interferon in the cells (12) The results also show that *under the experimental* conditions employed, a minimum of between 10 γ ml and 50 γ ml of hydrocortisone is required for inhibition of *interferon* production

To ascertain that the inhibitory activity of the media described in the above experiments really was due to interferon a series of experiments was performed

First the possibility that the inhibitory activity of the media could be due to interference by inactivated influenza virus was investigated employing material from the above experiment The media were submitted two consecutive *one hour cycles of centrifugation* at 40,000 r p m 144 000 g) in a Spinco Model L centrifuge and subsequently titrated It was *found that the high* speed centrifugation did not reduce the in-

hibitory activity of the suspension indicating that the inhibitor was not identical with inactivated influenza virus Titration before and after ultracentrifugation was also performed on material with a high inhibitory activity (Titre: 1/100). This medium was obtained considerably later in the experimental series from cultures incubated for a prolonged period of time, *i e* 30 hours after inoculation of virus In this case also it was found that two consecutive one hour cycles of centrifugation at 144,000 g did not reduce the inhibitory activity of the medium

Storage of the supernates from the ultracentrifuged tissue culture fluids at +4° C for more than 4 months did not decrease the inhibitory activity. Heating to 56° C for one hour and acidification at pH 2 for 24 hours were also without influence upon the inhibitory activity of tissue culture fluids The activity was completely lost, however, when the fluids were incubated for one hour at 36° C with 0 5 mg crystalline trypsin per ml.

These findings are all in agreement with the concept that the inhibitory activity of the tissue culture fluid is due to interferon (5)

The experiments described above thus show that preincubation with hydrocortisone inhibits the production of interferon in influenza A WS infected c e f cultures In a subsequent experiment the influence of hydrocortisone on production of influenza virus in c e f tissue cultures was investigated

C e f cultures preincubated for 24 hours with 250 γ hydrocortisone per ml and control cultures without hydrocortisone had the medium removed and were subsequently washed with 10 ml of Earle's BSS per culture The cultures were inoculated with influenza A WS virus as described above After one hour of virus adsorption at 36° C the inoculum was removed, the cultures were washed with 10 ml of Earle's BSS, maintenance medium was added and the incubation was continued at 36° C At intervals after the inoculation of virus, duplicate hydrocortisone pretreated cultures as well as control cultures were removed from the incubator The cells were scraped down and homogenized in the medium by vigorous pipetting and by rapid freezing and thawing three times Inactivation controls consisting of cell debris-medium mixture from hydrocortisone as well as control cultures inoculated with virus one hour previously were included in the main experiment The cell debris-medium mixtures collected during the experiment were stored at —60° C and +4° C, respectively, and after finishing the main experiment the samples stored at —60° C were titrated for infectivity and those stored at +4° C for haemagglutinin (12) The result of the infectivity titrations appears from Table 1

It can be seen that adsorption and penetration of influenza A WS virus into c e f cells occurred to the same extent whether the cells have been preincubated with hydrocortisone or not The production of infectious virus was low but the same in hydrocortisone treated cells and

controls, and neither hydrocortisone treated nor control cultures showed production of detectable amounts of haemagglutinin

The following experiment was designed to follow the production of interferon in c e f cultures, a production which had been *found to occur* under the experimental conditions described above

TABLE 1

The Influence of 24 Hours Preincubation with Hydrocortisone on the Production of Influenza A WS Virus in Chick Embryo Fibroblast Cultures

Pretreatment with		Virus titres (EID50*) hours after inoculation		
		1	24	48
Hydrocortisone		2 6	4 5	4 8
None		2 6	4 1	4 8
Inact controls	Hydrocortisone	2 6	1 7	0
	None	2 6	1 9	0

* Recorded as $\log_{10}$.

TABLE 2

The Production of Interferon in Chick Embryo Fibroblast Cultures Following Inoculation of Influenza A WS Virus

Hours	Number of plaques (average of Three cultures)						Titre of interferon§
	1	4	8	16	32	64	
18	63 0						0
24		0	28 3	40 3			10
30				0	6 0	38 7	57
48				0	6 7	39 7	57
Control	62 7						

* Reciprocal of dilutions

§ Recorded as reciprocal

C e f cultures were inoculated with influenza A WS virus at a multiplicity of 0 1 0 3 Following one hour of virus adsorption at 36° C the inoculum was removed, the cultures were washed with 10 ml of Earle's BSS and replenished with maintenance medium At intervals after the virus inoculation the media from duplicate cultures were harvested, pooled inactivated and subsequently tested for interferon Table 2 shows that detectable amounts of interferon were not produced until 18 hours after virus inoculation and also that by far the major part of the interferon production occurred between 18 and 30 hours after inoculation

In the following experiment the influence of preincubation for different periods of time with 100 γ ml of hydrocortisone on the production of interferon was investigated Except for varying preincubation periods the technique employed was identical with that described above Harvest and pooling of media from duplicate cultures were done 30 hours after

hibitory activity of the suspension indicating that the inhibitor was not identical with inactivated influenza virus. Titration before and after ultracentrifugation was also performed on material with a high inhibitory activity (Titre 1/100). This medium was obtained considerably later in the experimental series from cultures incubated for a prolonged period of time, i.e. 30 hours after inoculation of virus. In this case also it was found that two consecutive one hour cycles of centrifugation at 144,000 g did not reduce the inhibitory activity of the medium.

Storage of the supernates from the ultracentrifuged tissue culture fluids at +4° C for more than 4 months did not decrease the inhibitory activity. Heating to 56° C for one hour and acidification at pH 2 for 24 hours were also without influence upon the inhibitory activity of tissue culture fluids. The activity was completely lost, however, when the fluids were incubated for one hour at 36° C with 0.5 mg crystalline trypsin per ml.

These findings are all in agreement with the concept that the inhibitory activity of the tissue culture fluid is due to interferon (5).

The experiments described above thus show that preincubation with hydrocortisone inhibits the production of interferon in influenza A WS infected c.e.f. cultures. In a subsequent experiment the influence of hydrocortisone on production of influenza virus in c.e.f. tissue cultures was investigated.

C.e.f. cultures preincubated for 24 hours with 250 γ hydrocortisone per ml and control cultures without hydrocortisone had the medium removed and were subsequently washed with 10 ml of Earle's BSS per culture. The cultures were inoculated with influenza A WS virus as described above. After one hour of virus adsorption at 36° C the inoculum was removed, the cultures were washed with 10 ml of Earle's BSS, maintenance medium was added and the incubation was continued at 36° C. At intervals after the inoculation of virus, duplicate hydrocortisone pretreated cultures as well as control cultures were removed from the incubator. The cells were scraped down and homogenized in the medium by vigorous pipetting and by rapid freezing and thawing three times. Inactivation controls consisting of cell debris-medium mixture from hydrocortisone as well as control cultures inoculated with virus one hour previously were included in the main experiment. The cell debris-medium mixtures collected during the experiment were stored at —60° C and +4° C, respectively, and after finishing the main experiment the samples stored at —60° C were titrated for infectivity and those stored at +4° C for haemagglutinin (12). The result of the infectivity titrations appears from Table 1.

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In the following experiment the influence of preincubation for differ-

and pooling of media from duplicate cultures were *done 30 hours after*

inoculation of virus. The pools were inactivated and subsequently tested for interferon. Table 3 summarizes the results. It appears that only preincubation for 24 hours had any inhibitory effect on the production of interferon, an inhibition which in the present experiment was only partial. Cultures preincubated for 12 or 6 hours showed interferon titres identical with those of the control.

TABLE 3

The Influence of Hydrocortisone Preincubation for Varying Periods of Time on the Production of Interferon in Chick Embryo Fibroblast Cultures Inoculated with Influenza A WS Virus

Hours of preincubation with hydrocortisone 100γ/ml	Titre of interferon§
24	8
12	16
6	16
Control (no preincubation)	16

§ Recorded as reciprocal.

DISCUSSION

The present series of experiments originally arose from studies on hormonal influence upon viral interference (16). It was found that under the experimental conditions employed, detectable amounts of interferon appeared in the tissue culture fluids from c.e.f. cells inoculated with influenza A WS virus and that significantly less interferon was produced when the cultures had been preincubated with hydrocortisone (9, 13). This observation is in agreement with previous results obtained in this laboratory on the influence of hydrocortisone upon production of interferon in embryonated eggs inoculated with influenza virus (12). The observation is also in agreement with results obtained by *De Maeyer & De Maeyer* in tissue culture experiments. These authors found that steroid treatment reduced the production of interferon in a rat tumour cell line infected with Sindbis virus (10).

In the present experiments the influence of prolonged incubation of influenza A WS inoculated cultures were studied (Table 2) and it was shown that by far the major part of interferon production occurred between 18 and 30 hours after inoculation. Consequently, harvest of media approximately 30 hours after virus inoculation was employed in a subsequent series of experiments concerned with hormonal influence upon interferon production. These experiments are reported in a following paper (15).

The production of infectious influenza A WS virus in c.e.f. cells was found to be the same whether or not the cells had been treated with hydrocortisone (Table 1). This observation agrees well with previous experiments in this laboratory which showed infectivity titres of influenza virus grown in hydrocortisone treated chick embryos identical

with those of untreated controls. The finding (Table 1) that adsorption, penetration and growth of influenza A WS virus take place in hydrocortisone treated c.e.f. cells as it does in untreated cells rules out the possibility that the decreased interferon production in hydrocortisone treated cells could be due to a change in virus adsorption and multiplication in these cells.

The finding that hydrocortisone suppresses the yield of interferon without increasing the yield of infectious virus in c.e.f. cultures inoculated with influenza virus is an interesting observation in view of the effect of certain other inhibitors of interferon production. Treatment with actinomycin D or preinfection with parainfluenza 3 virus thus may result in an increased production of virus as well as a decreased production of interferon (2, 3).

Observations similar to those presented here, i.e. diminished production of interferon and unchanged production of virus has been made also by *De Maeyer & De Maeyer* in tissue cultures treated with a steroid configurated carcinogen 20-methylcholanthrene as well as with steroid hormones (10, 11). It seems therefore evident that certain steroids are able to reduce interferon production without a simultaneous increase in virus production.

The effects of steroid hormones at the cellular level are still obscure though considerable efforts have been directed towards the solution of this problem (1, 8). One may speculate that hydrocortisone exerts its effect by reducing the protein synthesizing abilities of the cells thus decreasing the production of interferon. If hydrocortisone besides having a marked effect on interferon synthesis also has a certain inhibitory effect upon synthesis of proteins constituting influenza virus particles, the diminished production of interferon need not necessarily result in an enhanced yield of virus (12). Indications of a suppression of virus multiplication by hydrocortisone have been observed in previous studies (6, 12) in which a delay of haemagglutinin synthesis was noted during the early phase of influenza virus infection in hydrocortisone treated chick embryos.

SUMMARY

Preincubation with 50 γ hydrocortisone per ml for 24 hours was found to reduce the production of interferon in influenza A WS infected chick embryo fibroblast tissue cultures, while preincubation with 10 γ hydrocortisone per ml had no influence on the interferon production.

Prolonged treatment with hydrocortisone was essential to decrease interferon production since preincubation with 100 γ hydrocortisone per ml for 24 hours diminished interferon production while preincuba-

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By

JORUNN ERIKSEN

Received 28 xii 64

In an earlier paper in this series (9) it was found that the chemical composition of the capsular polysaccharide from strains of *Klebsiella rhinoscleromatis*, *Klebsiella ozaenae*, *Klebsiella pneumoniae* and *Aerobacter (Klebsiella) aerogenes* was the same The polysaccharides contained galactose, mannose and uronic acid Another paper (18) dealing with the serological reactions of the same strains showed that they had the same serological specificity However the result of quantitative precipitin determinations in immune sera with homologous and heterologous antigens suggested that the antigens contained at least two different polysaccharides one of them reacting only in homologous serum The precipitin curves and the gel precipitations showed that the homologous reacting antigen must be present only in very small quantities The quantity must in fact be too small to have any influence on the data obtained by the chemical analysis

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Klebsiella pneumoniae type 1(A) showed that it did not contain galactose only D glucose L fucose and D glucuronic acid *Klebsiella ozaenae* type 4(D) contained galactose glucose mannose and uronic acid (20)

In 1955 *Wilkinson et al* (29) found that the extracellular polysaccharide of *Aerobacter aerogenes* A3 (S1) (*Klebsiella* type 54) contained D glucose L-fucose and an unidentified uronic acid In 1956 the com

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position of the extracellular polysaccharides of four *Aerobacter-Klebsiella* strains was investigated (7). The results of this examination showed that *Klebsiella* type 8, 26, 29 and 57 all contained galactose, mannose and uronic acid, but the types 8 and 26 contained glucose in addition

The acidic polysaccharide of *Aerobacter aerogenes* NCTC 8172 (*Klebsiella* type 64) was examined (2) and found to contain L rhamnose, D glucose, D-mannose and glucuronic acid Trace of mannuronic acid was also present Mannuronic acid was also found in *Aerobacter aerogenes* N C T C 418, which in addition contained D-glucose, D mannose and glucuronic acid (3)

From this survey, the only thing in common for the different types, seemed to be the content of uronic acid

This paper will deal with the purification of the capsular antigen isolated from *Klebsiella* type 3(C), and the structure of the acidic polysaccharide

PURIFICATION OF THE CAPSULAR POLYSACCHARIDE

The strains of *Klebsiella* type 3(C) were the same as used for earlier investigations (9), but two more strains, 270 60 and 1204, *Klebsiella pneumoniae* were added Table 1 contains the biochemical characteristics of these strains

TABLE 1

Biochemical Characteristics of the New Strains of Klebsiella pneumoniae *Type 3(C)*

Strains	Lactose	Glucose	Saccharose	I	M	V	C	Ur
270/60	A	A + G	A	—	+	—	+	+
1204	A	A + G	A	+	+	—	+	+

A Acid G Gas

The crude polysaccharides were prepared by cold water extraction of lactose agar cultures, as described previously (17) The product obtained after this method was used for further investigations The polysaccharide was dissolved in water and a solution of 2 per cent cetyl pyridinium chloride was added in drops The acidic polysaccharide was precipitated as a complex (25) The amount of cetyl pyridinium chloride was easily determined as a flocculation end point The precipitate was washed twice with distilled water, and 2N sodium chloride was added to break the complex between acidic polysaccharide and cetyl pyridinium chloride The acidic polysaccharide was dissolved, while the cetyl pyridinium chloride was precipitated The solution of acidic polysaccharide was precipitated by 3 volumes of 96 per cent ethanol, centrifuged, washed with 96 per cent ethanol and dried The polysaccharide was dissolved again in water and dialysed for 24 hr in running tap water to get rid of the last traces of sodium chloride After dialysing,



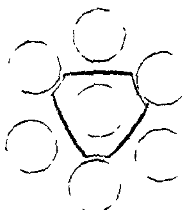


Fig 1 *Fig 2*

Fig 1 Immune serum anti type 3(C) in the central well The capsular acidic poly saccharide of six different strains all *Klebsiella* type 3(C) [illegible]

[illegible] polysaccharides from *Klebsiella pneumoniae* type 3(C) strain F 10 [illegible] as antigens

the polysaccharide was precipitated once more by 3 volumes of 96 per cent ethanol and some sodium acetate, left in the refrigerator till the following day, centrifuged in the cold, washed three times with 96 per cent ethanol and dried in vacuo above P_2O_5

The capsular polysaccharides of the six different strains, all belonging to *Klebsiella* type 3(C), were prepared in the same way As antigens they gave only one identical line by gel precipitation (10) with homologous and heterologous immune sera (Fig 1)

The supernatant after the precipitation of the acidic polysaccharide with cetyl pyridinium chloride was evaporated to a small volume, and a neutral polysaccharide was precipitated by 3 volumes of 96 per cent ethanol The precipitate was left in the refrigerator till the next day, centrifuged, washed with 96 per cent ethanol and dried above P_2O_5 in vacuo

1 g of crude material yielded about 850 mg of purified acidic polysaccharide, but only about 50 mg of neutral polysaccharide The neutral polysaccharide was used as an antigen in gel precipitation It reacted in homologous serum only, and was quite different from the type specific polysaccharide (Fig 2) The properties of the neutral polysaccharide will be discussed later

The type specific acidic polysaccharides of the six different strains were hydrolysed They all contained galactose, mannose and uronic acid Phosphorus was determined (21), but there was no phosphorus

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The crude polysaccharides were prepared by cold water extraction of lactose agar cultures, as described previously (17) The product obtained after this method was used for further investigations The polysaccharide was dissolved in water and a solution of 2 per cent cetyl pyridinium chloride was added in drops The acidic polysaccharide was precipitated as a complex (25) The amount of cetyl pyridinium chloride was easily determined as a flocculation end point The precipitate was washed twice with distilled water, and 2N sodium chloride was added to break the complex between acidic polysaccharide and cetyl pyridinium chloride The acidic polysaccharide was dissolved, while the cetyl pyridinium chloride was precipitated The solution of acidic polysaccharide was precipitated by 3 volumes of 96 per cent ethanol, centrifuged, washed with 96 per cent ethanol and dried The polysaccharide was dissolved again in water and dialysed for 24 hr in running tap water to get rid of the last traces of sodium chloride After dialysing,



Fig 1

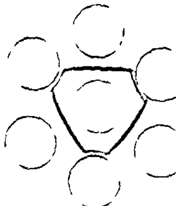


Fig 2

Fig 1 Immune serum anti type 3(C) in the central well The capsular acidic poly saccharide of six different strains all *Klebsiella* type 3(C) as antigens in the

Fig 2 1

as antigens

the polysaccharide was precipitated once more by 3 volumes of 96 per cent ethanol and some sodium acetate, left in the refrigerator till the following day, centrifuged in the cold washed three times with 96 per cent ethanol and dried in vacuo above P_2O_5

The capsular polysaccharides of the six different strains, all belonging to *Klebsiella type 3(C), were prepared in the same way* As antigens they gave only one identical line by gel precipitation (10) with homologous and heterologous immune sera (Fig 1)

The supernatant after the precipitation of the acidic polysaccharide with cetyl pyridinium chloride was evaporated to a small volume, and a neutral polysaccharide was precipitated by 3 volumes of 96 per cent ethanol The precipitate was left in the refrigerator till the next day, centrifuged, washed with 96 per cent ethanol and dried above P_2O_5 in vacuo

1 g of crude material yielded about 850 mg of purified acidic polysaccharide but only about 50 mg of neutral polysaccharide The neutral polysaccharide was used as an antigen in gel precipitation It reacted in homologous serum only and was quite different from the type specific polysaccharide (Fig 2) The properties of the neutral polysaccharide will be discussed later

The type specific acidic polysaccharides of the six different strains were hydrolysed They all contained galactose, mannose and uronic acid Phosphorus was determined (21), but there was no phosphorus

Fig 3

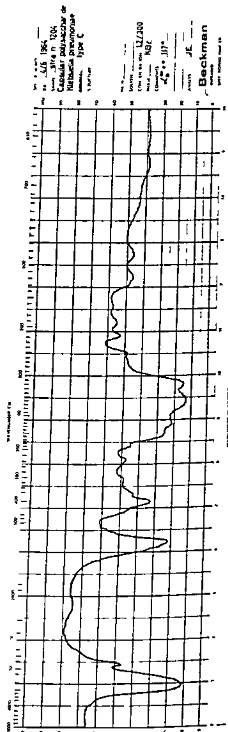


Fig 4

Fig 3 Infrared spectrum of acidic polysaccharide from *Klebsiella pneumoniae* type 3(C) strain F 10 N Y

Fig 4 Infrared spectrum of acidic polysaccharide from *Klebsiella pneumoniae* type 3(C) strain 1204

Fig 5

Fig 6

Fig 7

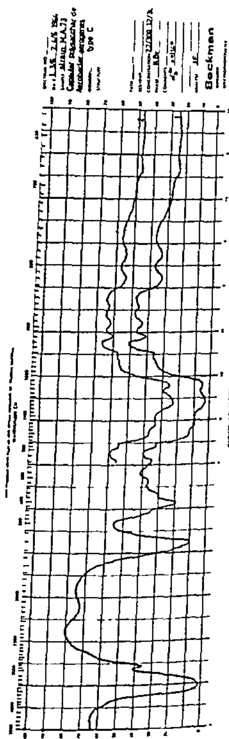


Fig 8

Fig 7. Infrared spectrum of acidic polysaccharide from *Klebsiella rhinoscleromatis* type 3(C) strain 92/04

Fig 8 Infrared spectrum of acidic polysaccharide from *Aerobacter (Klebsiella) aerogenes* type 3(C) M A 73

in any of them Nitrogen was determined by the micro-Kjeldahl method (21) Optical rotation was measured in a Hilger and Watts polarimeter M 412 (c 0 3 water) as Na salt (Table 2)

TABLE 2

Optical Rotation and Nitrogen Content of the six Acidic Polysaccharides

Acidic polysaccharides from strain	Optical rotation $[\alpha]_D^{20}$	Per cent nitrogen
K pneumoniae F 10 N Y	+ 112 3°	0 1
K pneumoniae 1204	+ 117 3°	0 7
K pneumoniae 2/0 60	+ 118 0°	0 0
K ozaenae 3828/60	+ 112 9°	0 1
K rhinoscleromatis 92/04	+ 113 0°	1 2
K aerogenes M A 73	+ 114 0°	0 2

The infrared absorption spectra of the polysaccharides were determined as the sodium salt in potassium bromide discs The concentration was 1 2 mg of polysaccharide/300 mg KBr Two spectra were also taken with the concentration of 2 2 mg of polysaccharide/300 mg KBr The infrared spectrophotometer used was Beckman I R 5A All spectra were identical (Fig 3 4, 5, 6, 7 and 8)

The acidic polysaccharide produced by *Klebsiella ozaenae* type 3(C) strain 3828/60 $[\alpha]_D^{20} = +112.9°$, $N_2 = 0.1$ per cent, was used for further investigation The reason why this strain was preferred, was that this culture gave the best yield of polysaccharide

PARTIAL HYDROLYSIS OF THE ACIDIC POLYSACCHARIDE

Because the acidic polysaccharide contained uronic acid, sulphuric acid was always used for the hydrolysis The hydrolysate was neutralized with barium carbonate and centrifuged The supernatant was then treated with Amberlite Resin I R 120 (H⁺) and concentrated to a small volume ready for chromatography Descending chromatography on Whatman paper No 1 was used Solvent developers were

1
2
3
4 5v 5v 1v 3v
5
6 Butan 1 ethanol water ammonia 40v 10v 49v 1v

Most of the chromatograms were run twofold for spraying with two different reagents As colour reagents for identification of the sugar spots were used a) aniline hydrogen phthalate (27) and b) silver nitrate-sodium hydroxide (28)

21

Fig 7

Fig 8

Fig 7. Infrared spectrum of acidic polysaccharide from *Klebsiella rhinoscleromatis* type 3(C) strain 92 04

Fig 8. Infrared spectrum of acidic polysaccharide from *Aerobacter (Klebsiella) aerogenes* type 3(C) M A 73

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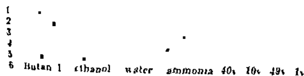
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K rhinoscleromatis 92/04	+ 113 0°	1 2
K aerogenes NA 73	+ 114 0°	0 2

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Most of the chromatograms were run twofold for spraying with two different reagents As colour reagents for identification of the sugar spots were used a) aniline hydrogen phtalate (23) and b) silver nitrate sodium hydroxide (28)

The same amount of polysaccharide was hydrolysed for 3 hr at 100° with different concentrations of sulphuric acid as 2N, 1N, 0 1N and 0 01N The hydrolysates were neutralized and submitted to paper chromatograms in solvent 1

The results were·

2N sulphuric acid galactose, mannose and uronic acid

1N sulphuric acid: galactose, mannose and uronic acid, but also a fraction with R_f-value between those for uronic acid and galactose This could be a disaccharide

0 1N sulphuric acid. This gave the same fractions as the hydrolysis with 1N sulphuric acid, but on a smaller scale

Most of the polysaccharide was unhydrolysed

0 01N sulphuric acid Most of the polysaccharide was still unhydro lysed, but a small amount of mannose could be seen

The preliminary hydrolysis indicated that 1N sulphuric acid probably would give a disaccharide This concentration was therefore used when a big portion was hydrolysed for cellulose column chromatography

1 g of polysaccharide was hydrolysed with 45 ml of 1N sulphuric acid for 3 hr at 100° The hydrolysate was treated in the usual way before evaporation to dryness to obtain the weight which was 880 mg The glass column for chromatography was 500 mm long and had an inside dia meter of 50 mm The bottom was layered with glass fibers The column was packed dry with Whatman standard grade cellulose powder to 450 mm height The top was covered with a porous filter paper disk The column was soaked with the irrigant which was solvent 4 The sample was dissolved in the irrigant and applied to the top of the column The irrigant was then started through the column Fractions of 10 ml were collected by an automatic fraction collector at a rate of three fractions an hour Spots of the fractions were applied to a paper chromatogram, developed in solvent 2 and sprayed with silver nitrate-sodium hydroxide

Five different fractions were collected from the column and identified by paper chromatography, 200 mg mannose, 150 mg galactose, 100 mg disaccharide, 60 mg uronic acid and 100 mg aldobiuronic acid

Optical rotation of the mannose fraction was $[\alpha]_D^{20} = +12\ 7°$ (c 1 3 water) The mannose was characterized (24) as 2,3 5,6-Di-O-isopropylidene-α-D-mannofuranose with m p 120°, alone and mixed with an authentic sample

Optical rotation of the galactose fraction was $[\alpha]_D^{02} = +50\ 0°$ (c 1 0 water) The galactose was characterized (31) as β-D-galactopyranose penta acetate with m p 139°, alone and mixed with an authentic sample

The colour of the solution of the disaccharide was too dark to give any information about the optical rotation A small portion was hydrolysed with 2N sulphuric acid at 100° for 4 hr and applied to a paper chromatogram which was developed in solvent 2 The disaccharide contained mannose and galactose

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followed by De Acidite FF (CO3 ...
sulphuric acid for 3 hr at 100° and applied to a paper chromatogram with standards of mannitol and galactitol The chromatogram was ... hr and dipped in *benzidine hydrochloride*

The optical rotation of the uronic acid ... cellulose column was $[\alpha]_D^{20} = +26.8°$ (c 0.4 water) On paper chromatograms developed in solvent 5 for 17 hr and in solvent 1 for 18 hr the fraction had the same R_f value as galacturonic acid Spraying with silver nitrate sodium hydroxide showed no spot of lactone The fraction had the same mobility as galacturonic and glucuronic acid by *paper ionophoresis* in acetate buffer pH = 5

The *uronic acid* fraction was converted to the methyl ester methyl glycoside by refluxing for 6 hr with 2 per cent methanolic hydrogen *chloride* Excess of hydrogen chloride was removed by repeated distillations *with dry methanol in vacuo The residue* was kept over night above *potassium* hydroxide in vacuo The next day it was *dissolved in dry* tetrahydro furan and dry ether (4: 1v) *and lithium* aluminium hydride was added *in small* portions during *one hour at room temperature* Magnetic stirring was used all the time After the *addition* of lithium aluminium hydride the stirring was continued for another hour, and then the solution was brought to 80° for *half an hour* Excess of lithium aluminium hydride was destroyed by addition of water The solution was filtered and deionized with Amberlite Resin I R 120 (H) and De Acidite FF (CO_3) After evaporation to dryness the residue was hydrolysed with 2N sulphuric *acid for* 3 hr and applied to paper chromatography in solvent 1 for 48 hr The chromatogram showed galactose which *indicated* that the uronic acid was *galacturonic* acid

The aldobiuronic acid isolated from the cellulose column had an optical rotation $[\alpha]_D^{20} = +100°$ Paper ionophoresis in acetate buffer, pH = 5 showed a *mobility of 0 7* compared with the galacturonic acid The aldobiuronic acid was hydrolysed *for* 7 hr with 2N sulphuric acid applied to a paper chromatogram and developed in solvent 2 It contained mannose and a uronic acid *but* still some unhydrolysed a dobiuronic acid

To identify the uronic acid *linked* to the mannose the aldobiuronic acid was refluxed for 6 hr with *2 per cent* methanolic hydrogen chloride and the resulting methyl ester *methyl glycoside* was reduced with lithium aluminium hydride *in the same* way as *for* the *uronic acid* fraction Hydrolysis *of the methyl bioside* with 2N *sulphuric* acid,

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To obtain information about the reducing end of the disaccharide, it as reduced for 5 hr with sodium borohydride in water under an mosphere of carbon dioxide to maintain a neutral solution After the duction, the solution was deionized with Amberlite Resin I R 120 (H^+) llowed by De-Acidite FF (CO_3), concentrated, hydrolysed with 2N [illegible] at 100° and applied to a paper chromatogram

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some mannose left No decrease in the amount of galactose or uronic acid could be seen from the chromatogram A quantitative determination of mannose (30) in the polysaccharide before and after oxidation gave a more significant figure of the difference. It contained 25.1 per cent mannose before and 4 6 per cent after oxidation

ALKALI TREATMENT OF THE ACIDIC POLYSACCHARIDE

Before methylation, the polysaccharide was treated with 1N sodium hydroxide in an atmosphere of nitrogen, to see if it was resistant to alkali The polysaccharide was dissolved in water 2N sodium hydroxide was added until the final concentration of sodium hydroxide was 1N N_2 was bubbled through the solution all the time At different intervals, 0 5, 2, 4, 6 hr and 1, 2 and 3 days, aliquots were neutralized with 2N hydrogen chloride The neutral solution was used as antigen for a gel precipitation From the gel precipitation, there was no difference to see between polysaccharides, untreated or treated with alkali for three days

METHYLATION OF THE ACIDIC POLYSACCHARIDE

For the methylation of the polysaccharide, Haworth's method with sodium hydroxide and dimethylsulphate was used (14) The polysaccharide was dissolved in water Sodium hydroxide and dimethylsulphate were added alternately every tenth minute at room temperature Vigorous stirring was used, and continued for 2 hr. after the reagents were added The solution was neutralized with acetic acid, dialysed in running tap water for 24 hr and freezedried The procedure was repealed four times After the second methylation the partly methylated polysaccharide precipitated from the acidic solution After four methylations, the methylated polysaccharide was centrifuged off the acidic solution dissolved in water, dialysed and freezedried The methylated product was refluxed for 5 hr with a 5 per cent solution of hydrogen chloride in dry methanol The solution was neutralized with excess of silver carbonate and centrifuged The supernatant was evaporated to dryness hydrolysed with 2N sulphuric acid for 5 hr and applied to a paper chromatogram in solvent 6 for 17 hr

Six different spots were seen on the chromatogram The R_f-values in solvent 6 were 0 77 0 68, 0 58, 0 48, 0 23 and 0 14 R_f-value for glucose in the same solvent was 0 17 When the solution was deionized with De-Acidite FF (CO_3) before chromatography, two of the spots (R_f = 0 14 and 0 23) disappeared which indicated that they belonged to the methylated uronic acid(s)

Two other spots (R_f = 0 48 and 0 68) were identified as 2,4,6-tri-O-*methyl*-D-galactose and 2,3,6-tri-O methyl-D-mannose, both compared

showed a content of mannose and galactose by paper chromatography in solvent 1 for 40 hr. The galactose must be the reduction product of the uronic acid, and the aldobiuronic acid identified as galacturonic acid-mannose.

PERIODATE OXIDATION OF THE ACIDIC POLYSACCHARIDE

The acidic polysaccharide was oxidized by sodium periodate at room temperature in the dark (11, 19, 13). Aliquots of the reaction mixture were periodically removed and analysed for periodate consumption. The results were plotted against time. At the same time mannitol was oxidized with the same periodate solution, and the consumption of periodate was determined with the same reagents as a control. It was found that 1 mol of mannitol had a consumption of 4.96 mol periodate. The results for the polysaccharide are in Table 3.

TABLE 3

Consumption of Periodate by Oxidation of the Acidic Polysaccharide

Time in hour	0.5	1.5	5	24	48	72	96	168
Mol periodate *per mol* anhydrosugar	*0.10*	*0.21*	*0.30*	*0.33*	*0.33*	*0.33*	*0.33*	*0.33*

The oxidation was complete after 5 hr and no overoxidation could be seen, even after 7 days. Whether acid was formed during oxidation, was examined in an aliquot of the reaction mixture. Excess of periodate was removed by addition of ethylene glycol. After 10 min the solution was titrated against 0.01N sodium hydroxide potentiometrically by means of a glass electrode and a pH-meter. Mannitol was used as a control, and 1 mol of mannitol was found to give 4 mol of formic acid during the oxidation. However, oxidation of the polysaccharide did not produce any acid.

Formation of formaldehyde during the oxidation was determined by the specific reaction of formaldehyde with chromotropic acid (4,5-dihydroxy-2,7-naphtalene disulphonic acid) (22). The calibration curve was derived from the oxidation of mannitol. 1 mol mannitol gives by oxidation 2 mol formaldehyde. Aliquots were taken from the oxidation mixture after 5, 15 and 30 min. and 1, 2, 3, 4, 5 and 24 hr., but no formaldehyde was found to be formed. The polysaccharide, oxidized for 24 hr was dialysed in running tap water for 24 hr. The solution was evaporated by freezedrying. Some of the oxidized polysaccharide was hydrolysed with 2N sulphuric acid for 3 hr. applied to a paper chromatogram and developed in solvent 1 for 30 hr. The chromatogram showed that the amount of mannose had decreased by oxidation, but there was still

with lithium aluminium hydride Acidic hydrolysis of the ... gave mannose and galactose The galactose was the reduction product of the uronic acid The aldobiuronic acid was galacturonic acid-mannose

The neutral disaccharide isolated from the cellulose column was shown to contain mannose and galactose The disaccharide was reduced by sodium borohydride and acid hydrolysis of the disaccharide alcohol gave galactose and mannitol which showed that the disaccharide was galactosyl mannose

Periodate oxidation of the polysaccharide showed a consumption of 0 33 mol periodate per mol of anhydrosugar, but neither formaldehyde nor formic acid was formed during the oxidation Formaldehyde (27) is produced from polysaccharides containing hexofuranose units Formic acid is librated by oxidation of polysaccharides containing hexopyranose units, either from the non reducing end group or units linked 1 6 Hexopyranose units linked 1,3 or engaged in branching of 1,3,6 – 1 2,4 or 1 3 4 types are resistant to periodate attack Since this polysaccharide was not resistant to oxidation, and no formic acid and formaldehyde were formed, some of the linkages must be 1,4 Because the consumption was only 0 33 mol periodate per mol anhydrosugar, about ⅔ of the linkages were resistant to oxidation and therefore could be 1 3 However, the possibility of some branching cannot be excluded Hydrolysis and quantitative determination of mannose before and after oxidation showed a decrease from 25 per cent to 5 per cent which indicated that mannose must be linked both 1,4 and 1,3 No overoxidation could be seen, even after 7 days, in spite of the fact that the polysaccharide contained uronic acid This indicated that the uronic acid could not be the non reducing end group or linked through the 2 position

Before methylation, which involves treatment of the polysaccharide with equivalent amounts of 30 per cent of sodium hydroxide and dimethylsulphate could take place, the effect of alkali had to be investigated When the polysaccharide was treated with 1N sodium hydroxide in an atmosphere of nitrogen for three days, no effects could be seen It still reacted as antigen with the antiserum

The polysaccharide was methylated four times after the Haworth s method After the second methylation, the partly methylated polysaccharide started to precipitate from the acidic mixture This is specially true when the polysaccharide contains uronic acid residues (26)

After hydrolysis of the methylated polysaccharide, the isolation of 2 3 6-tri O methyl D mannose and 2,4,6 tri O-methyl-mannose was in agreement with the oxidation results Mannose was linked 1,3 and 1,4 The isolation of 2 4,6-tri-O-methyl D-galactose showed that galactose was linked 1,3 The identification of 2,3,4 6 tetra O methyl-mannose indicated mannose to be the non reducing end group Mannose was also

with standards.* The spot with $R_f = 0.77$ was identified as 2,3,4,6-tetra-O-methylmannose. The spot with $R_f = 0.58$ did not migrate in 0.1 M borate buffer, pH = 10, and it could be 2,4,6-tri-O-methylmannose.

DISCUSSION

From an earlier paper it was suggested that the assumed capsular polysaccharide could be contaminated with another antigen which reacted in homologous antiserum only. This investigation showed that by means of cetyl pyridinium chloride, the crude polysaccharide was separated in two different fractions. The acidic polysaccharide was the type specific antigen. The neutral polysaccharide was the antigen which reacted only in homologous antiserum. Chemical and serological investigations of the neutral polysaccharide will be dealt with later. The capsular acidic polysaccharide has been investigated more closely, and the results will be discussed here.

The acidic polysaccharide from six different strains, *Klebsiella pneumoniae* strain F 10 N.Y., strain 1204 and strain 270/60, *Klebsiella ozaenae* strain 3828/60, *Klebsiella rhinoscleromatis* strain 92/04 and *Klebsiella aerogenes* strain M.A. 73, all type 3(C) were isolated in the same way. They showed identical serological reactions. The optical rotations were very close, and the infrared spectra were identical. By paper chromatography they all showed to contain galactose, mannose and uronic acid.

For a more detailed investigation of the acidic polysaccharide, *Klebsiella ozaenae* strain 3828/60 was chosen. By column chromatography D-galactose and D-mannose were isolated and identified as β-D-galactopyranose penta-acetate and 2,3: 5,6-Di-O-isopropylidene-α-D-mannofuranose.

The uronic acid was identified as galacturonic acid by glycosidation and esterification with methanolic hydrogen chloride, reduction with lithium aluminium hydride, hydrolysis, and paper chromatography which then gave galactose.

The linkages in the polysaccharide were not all of the same kind since two disaccharides were isolated. One of the disaccharides was an aldobiuronic acid. This type of disaccharide is relatively resistant to acid hydrolysis (6) and will survive acid hydrolysis under conditions where other oligosaccharides will be completely hydrolysed. Isolation of an aldobiuronic acid did not tell anything about the type of linkage in the disaccharide, but it will give informations about the sequence, which is generally assumed to be uronic acid-aldose (27). The aldobiuronic acid from this polysaccharide was hydrolysed to mannose and uronic acid

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* Grateful thanks are due to dr. S. A. Barker, Chemistry Department of Birmingham University, for valuable standards of methylated sugar.

Refluxing the aldobiuronic acid with 2 per cent hydrogen chloride in dry methanol gave the methylester methylglycoside which was reduced with lithium aluminium hydride. Acidic hydrolysis of the methylbioside gave mannose and galactose. The galactose was the reduction product of the uronic acid. The aldobiuronic acid was galacturonic acid-mannose.

The neutral disaccharide isolated from the cellulose column was shown to contain mannose and galactose. The disaccharide was reduced by sodium borohydride and acid hydrolysis of the disaccharide alcohol gave galactose and mannitol which showed that the disaccharide was galactosyl mannose.

Periodate oxidation of the polysaccharide showed a consumption of 0.33 mol periodate per mol of anhydrosugar, but neither formaldehyde nor formic acid was formed during the oxidation. Formaldehyde (27) is produced from polysaccharides containing hexofuranose units. Formic acid is librated by oxidation of polysaccharides containing hexopyranose units, either from the non reducing end group or units linked 1,6. Hexopyranose units linked 1,3 or engaged in branching of 1,3,6, 1,2,4 or 1,3,4 types are resistant to periodate attack. Since this polysaccharide was not resistant to oxidation, and no formic acid and formaldehyde were formed, some of the linkages must be 1,4. Because the consumption was only 0.33 mol periodate per mol anhydrosugar, about ⅔ of the linkages were resistant to oxidation and therefore could be 1,3. However, the possibility of some branching cannot be excluded. Hydrolysis and quantitative determination of mannose before and after oxidation showed a decrease from 25 per cent to 5 per cent which indicated that mannose must be linked both 1,4 and 1,3. No overoxidation could be seen, even after 7 days, in spite of the fact that the polysaccharide contained uronic acid. This indicated that the uronic acid could not be the non reducing end group or linked through the 2 position.

Before methylation, which involves treatment of the polysaccharide with equivalent amounts of 30 per cent of sodium hydroxide and dimethylsulphate, could take place, the effect of alkali had to be investigated. When the polysaccharide was treated with 1N sodium hydroxide in an atmosphere of nitrogen for three days, no effects could be seen. It still reacted as antigen with the antiserum.

The polysaccharide was methylated four times after the Haworth's method. After the second methylation, the partly methylated polysaccharide started to precipitate from the acidic mixture. This is specially true when the polysaccharide contains uronic acid residues (26).

After hydrolysis of the methylated polysaccharide, the isolation of 2,3,6-tri-O-methyl D-mannose and 2,4,6-tri-O-methyl mannose was in agreement with the oxidation results. Mannose was linked 1,3 and 1,4. The isolation of 2,4,6-tri-O-methyl D-galactose showed that galactose was linked 1,3. The identification of 2,3,4,6-tetra-O-methyl mannose indicated mannose to be the non reducing end group. Mannose was also

preferentially released on treatment of the polysaccharide with 0 01N sulphuric acid for 3 hr at 100°

How the uronic acid and the aldobiuronic acid were linked was difficult to interpret, because uronic acids are seldom completely methylated (6)

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Acta path. et microbiol. scandinav. 64, 362–366, 1965

Statens Seruminstitut, Kopenhagen, Dänemark

SUPPLEMENT ZUM KAUFFMANN-WHITE-SCHEMA (VIII)

Von

F. Kauffmann

Eingegangen 5.1.65

Diese Veröffentlichung ist das 4. Supplement zum *Kauffmann-White-Schema* in dem Buche *„Die Bakteriologie der Salmonella-Species“* (*F. Kauffmann* 1) und das 8. Supplement zu der Review *„Das Kauffmann-White-Schema“* (*F. Kauffmann* 2). Es enthält 33 neue *Salmonella-species* und 3 Varianten des *sub-genus* I, die im Laufe des Jahres 1964 festgestellt wurden und in der folgenden Tabelle angegeben sind.

Bevor wir aber hierauf näher eingehen, seien einige Bemerkungen über *„Paratyphus C“* vorausgeschickt. In dem Abschnitte „Paratyphus C“ auf Seite 264–265 des Buches *“The World Problem of Salmonellosis”* (herausgegeben von *E. van Oye*) hat *H. Seeliger*[1] eine falsche Darstellung des „Paratyphus C“, d. h. der menschlichen Erkrankung, die durch *S. paratyphi-C* verursacht wird, gegeben. Er schreibt nämlich wörtlich folgendes:

„Zu den Erregern des Paratyphus C zählen zwei eng verwandte *Salmonella*-Keime (*S. paratyphi C = S. hirschfeldii* und *S. cholerae-suis var. kunzendorf*). Hiervon abzugrenzen sind der Enteritiserreger *S. cholerae-suis* und die zahlreichen übrigen Serotypen der C-Gruppe.“

Hierzu muss folgendes gesagt werden: Der Erreger des „Paratyphus C“ ist nur die *Salmonella-species S. paratyphi-C*, also *nicht S. cholerae-suis var. kunzendorf*. Diese Variante darf nicht von der *species S. cholerae-suis* abgetrennt werden, da beide eine Einheit bilden, und zwar nicht nur in bakteriologischer, sondern auch in klinischer, pathogenetischer und epidemiologischer Hinsicht.

Der alleinige Erreger von „Paratyphus C“ (*S. paratyphi-C*) wird von Mensch zu Mensch übertragen und verursacht meist ein paratyphöses Krankheitsbild, ist aber oft nur sekundärer Erreger bei anderen Erkrankungen wie Malaria. Mit dem Schwein hat *S. paratyphi-C* nichts zu tun, in völligem Gegensatze zu der *species S. cholerae-suis* und ihrer Variante *S. cholerae-suis var. kunzendorf*, die beide ihren Standort im Schwein haben. Diese Erreger verursachen beim Menschen meist eine akute Enteritis, oft mit septischen Fokalprozessen, zuweilen aber auch ein paratyphöses Krankheitsbild wie auch andere Enteritis-Erreger der *Salmonella*-Gruppe.

1 (sich auf Publikationen von *Bader* und *Habs* stützend).

Im Gegensatz zu Infektionen mit *S paratyphi C* werden *S choleræ suis*-Infektionen sowie Erkrankungen durch *S choleræ suis var kunzendorf* nicht von Mensch zu Mensch übertragen, sondern sind meist Nahrungsmittelvergiftungen.

Die beiden species *S paratyphi C* einerseits und *S choleræ suis* (inclusive *S choleræ suis var kunzendorf*) andererseits sind also bakteriologisch, serologisch verschieden, sie verursachen verschiedene Erkrankungen und haben auch eine verschiedene Epidemiologie.

S paratyphi C (= 6 7 Vi) und *S choleræ suis* (= 6 7) haben nicht nur verschiedene Korper-Antigene, sondern auch verschiedene Geissel-Antigene, da beide Phasen (c 1 5) trotz der vereinfachten Formel ver

durch 2 ver

wenn

para

typhus

B durch *S paratyphi B* verursacht werden, ist *Paratyphus C* nur durch *S paratyphi C* bedingt.

Im folgenden sind einige vergärungsmässige Abweichungen, die in der Tabelle nicht angegeben sind, erwähnt:

S nor lufer bildete Indol.
S stegburg var sardinia war negativ in Ammonium-Citrat.
S laltenkamp spaltete Lactose nach 1 Tage.
S alexanderplat spaltete Salicin nach 1 Tage.

Liste der Salmonella Species des Sub-Genus I von 1964

Salmonella alexanderplat = 47 z_{38} —
Salmonella amounderness — 3 10 i 1 5
Salmonella bochum = 4 5 12 r L (4 5 12 r l w)
Salmonella bonames = 17 a 1 2
Salmonella bron — 13,22 G — (13 22 g m —) Wurde als monophasische Variante von S *bron* 13 22 g m e n z_{15} betrachtet
Salmonella cannstatt 1 3 19 G — (1,3 19 m t —)
Salmonella dembe = 35 d L (35 d l w)
Salmonella dresden — 28 c e n x
Salmonella etterbeek — 11 $z_4 z_{23}$ e n z_{15}
Salmonella gnesta — 1 3 19 b 1 5 Die Kultur enthielt ausserdem eine R-Phase
Salmonella gra Roschka & Roschka = 43 a 1 2 Z Hyg 150 94 Ja 1964
Salmonella hillegersberg = (9) 46 z_{35} 1 5
Salmonella inglis = (9) 46 z_0 e n x
Salmonella joenkoeping = 4 5 12 G — (4 5 12 g s t —)
Salmonella kihi = 1 42 z_{35} 1 6

Acta path et microbiol scandinav 64 362–366, 1965

Statens Seruminstitut, Kopenhagen, Danemark

SUPPLEMENT ZUM KAUFFMANN-WHITE-SCHEMA (VIII)

Von

F KAUFFMANN

Eingegangen 9 1 65

Diese Veroffentlichung ist das 4 Supplement zum *Kauffmann-White-Schema* in dem Buche *„Die Bakteriologie der Salmonella Species'* (*F Kauffmann* 1) und das 8 Supplement zu der Review *„Das Kauffmann White-Schema"* (*F Kauffmann* 2) Es enthalt 33 neue *Salmonella-species* und 3 Varianten des *sub-genus* I, die im Laufe des Jahres 1964 festgestellt wurden und in der folgenden Tabelle angegeben sind

Bevor wir aber hierauf naher eingehen, seien einige Bemerkungen uber *„Paratyphus C"* vorausgeschickt In dem Abschnitte „Paratyphus C" auf Seite 264–265 des Buches *"The World Problem of Salmonellosis'* (herausgegeben von *E van Oye*) hat *H Seeliger*[1] eine falsche Darstellung des „Paratyphus C", d h der menschlichen Erkrankung, die durch *S paratyphi-C* verursacht wird, gegeben Er schreibt namlich wortlich folgendes

„Zu den Erregern des Paratyphus C zahlen zwei eng verwandte *Salmonella*-Keime (*S paratyphi C* = *S hirschfeldii* und *S cholerae-suis var kunzendorf*) Hiervon abzugrenzen sind der Enteritiserreger *S cholerae suis* und die zahlreichen ubrigen Serotypen der C-Gruppe'

Hierzu muss folgendes gesagt werden Der Erreger des „Paratyphus C" ist nur die *Salmonella-species S paratyphi-C*, also *nicht S cholerae-suis var kunzendorf* Diese Variante darf nicht von der *species S cholerae-suis* abgetrennt werden, da beide eine Einheit bilden, und zwar nicht nur in bakteriologischer, sondern auch in klinischer, pathogenetischer und epidemiologischer Hinsicht

Der alleinige Erreger von „Paratyphus C' (*S paratyphi-C*) wird von Mensch zu Mensch ubertragen und verursacht meist ein paratyphoses Krankheitsbild, ist aber oft nur sekundarer Erreger bei anderen Erkrankungen wie Malaria Mit dem Schwein hat *S paratyphi-C* nichts zu tuen, in volligem Gegensatze zu der *species S cholerae-suis* und ihrer Variante *S cholerae suis var kunzendorf*, die beide ihren Standort im Schwein haben Diese Erreger verursachen beim Menschen meist eine akute Enteritis, oft mit septischen Fokalprozessen zuweilen aber auch ein paratyphoses Krankheitsbild wie auch andere Enteritis-Erreger der *Salmonella* Gruppe

1 (sich auf Publikationen von *Bader* und *Habs* stutzend)

Acta path et microbiol scandinav. 64, 367–372, 1965

Statens Seruminstitut, Kopenhagen, Danemark

WEITERE *SALMONELLA*-SPECIES DER SUB-GENERA II, III UND IV

Von

F Kauffmann

Eingegangen 4 1 65

In Fortsetzung einer fruheren Mitteilung „*Weitere Salmonella-species des sub genus II* (1963)" werden im folgenden die im Jahre 1964 festgestellten *species der sub-genera* II, III und IV beschrieben Wie bereits fruher gesagt, „wird es wahrscheinlich bald zweckmassig sein, die ‚*atypischen sub-genus II-species*" in einem besonderen *sub-genus* zu vereinen" Dieses erfolgte durch einen Vortrag auf der Tagung der osterreichischen Gesellschaft fur Mikrobiologie in Graz, im September 1964 Hier wurde ein neues *sub-genus* IV aufgestellt und umfasste die fruher als „*atypische sub-genus II-species*" bezeichneten Kulturen Die Differentialdiagnose zwischen den 4 aufgestellten *sub-genera* des *genus Salmonella* erfolgte nach folgendem Schema.

	I	II	III	IV
Dulcit	+	+	—	—
Lactose	—	—	+ oder ×	—
Salicin	—	—	—	+
Gelatine	—	+	+	+
Malonat	—	+	+	—
d Tartrat	+	— oder ×	— oder ×	— oder ×
KCN	—	—	—	+

× = spat und unregelmassig positiv

Gelatine wird von den typischen *species* des *sub-genus* I nicht verflussigt, dagegen von den *sub-genus* II-, III- und IV-*species* Die Gelatine

Ferner hat der Verfasser im Jahre 1959 Dr *Ingeburg Bohlck*, welche *S. kiel* = 1,2,12: g,p: — publizierte, gebeten, in ihrer Arbeit folgende Satze zu schreiben:

„*Kauffmann* ausserte in einer schriftlichen Mitteilung seine Ansicht, dass der neue Typ, der sich biochemisch wie *S dublin* verhalte, in derselben Weise genetisch mit *S. dublin* (1,9,12: gp) zusammenhangen konne wie *S. paratyphi A* (1,2,12: a) mit *S. sendai* (1,9,12. a: 1,5) Zwischen den beiden letztgenannten Typen bestehe neben dem phylogenetischen auch ein kultureller und klinischer Zusammenhang (*Kauffmann* 4). *Kauffmann* ist der Meinung, dass sowohl *S paratyphi A* aus *S. sendai* als auch der neue Typ aus *S dublin* durch Verlust des O-Antigens 9 entstanden sein konnten. Fur die Wahrscheinlichkeit, dass es sich um Verlustvarianten handelt, sprechen auch die Vermutungen von *White*, der monophasische Stamme als Verlustvarianten diphasischer Typen ansah"

Diese Vermutung des Verfassers wurde spater durch Dr *I Bohlck* bestatigt, da sie im Jahre 1964 mitteilte, dass der *S kiel*-Ausscheider unter 10 positiven Befunden, die seit 1958 erhoben werden konnten, zweimal *S dublin* aufwies Eine Mitteilung hieruber befindet sich im Archiv fur Hygiene und Bakteriologie im Druck [1] Diese Befunde machen es sehr wahrscheinlich, dass der Bacillentrager mit *S dublin* infiziert war, unde dass dann spater in seinem Korper die Verlustvariante *S kiel* entstanden ist

ZUSAMMENFASSUNG

In einem Supplement zum *Kauffmann-White-Schema* werden 33 neue *Salmonella-species* und 3 Varianten des *sub-genus* I, die im Laufe des Jahres 1964 festgestellt wurden, mitgeteilt Die neuen *species* der *subgenera* II, III und IV sind in einer folgenden Mitteilung enthalten

Bis Ende des Jahres 1964 waren ca 950 *Salmonella-species* der *subgenera* I, II und IV festgestellt worden Wenn hierzu die Arizona-Kulturen (*sub-genus* III) gerechnet werden, kommen wir auf eine Zahl von uber 1000 *Salmonella-species*

LITERATUR

Bohlck, I „Ein neuer Salmonellatyp *S kiel* (1 2 12 g p —) Arch Hyg und

Species' (Munksgaard, Kopen-

Kauffmann, F (2) , Das Kauffmann White-Schema in Ergebnisse der Mikrobiologie, Immunitatsforschung und Experimentellen Therapie *30* 160-216 1957 (Springer Verlag Berlin Gottingen-Heidelberg)

Kauffmann, F (3). „Supplement zum Kauffmann White Schema (VII)' Acta path et microbiol scandinav *61* 583 587 1964

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1 (Arch Hyg und Bakter *149* 185 187, 1965)

Acta path et microbiol scandinav 64, 367 372, 1965

Statens Seruminstitut, Kopenhagen, Danemark

WEITERE *SALMONELLA*-SPECIES DER SUB-GENERA II, III UND IV

Von

F Kauffmann

Eingegangen 4 1 65

In Fortsetzung einer fruheren Mitteilung „*Weitere Salmonella-species des sub genus II* (1963)" werden im folgenden die im Jahre 1964 festgestellten *species* der *sub-genera* II, III und IV beschrieben Wie bereits fruher gesagt, „wird es wahrscheinlich bald zweckmassig sein, die ‚*atypischen sub genus II-species*' in einem besonderen *sub-genus* zu vereinen" Dieses erfolgte durch einen Vortrag auf der Tagung der osterreichischen Gesellschaft für Mikrobiologie in Graz, im September 1964 Hier wurde ein neues *sub genus* IV aufgestellt und umfasste die fruher als „*atypische sub-genus II-species*' bezeichneten Kulturen Die Differentialdiagnose zwischen den 4 aufgestellten *sub genera* des genus *Salmonella* erfolgte nach folgendem Schema

	I	II	III	IV
Dulcit	+	+	—	—
Lactose	—	—	+ oder ×	—
Salicin	—	—	—	+
Gelatine	—	+	+	+
Malonat	—	+	+	—
d Tartrat	+	— oder ×	— oder ×	— oder ×
KCN	—	—	—	+

× = spat und unregelmassig positiv

Wie man aus obigem Schema ersieht ist die biochemische Differen- [illegible] ie sowie

[illegible] species der sub-genera I und II meist gespalten, nicht aber von den *species* der *sub genera* III und IV

Lactose wird von den *species* des *sub-genus* III (*Salmonella arizonae*) prompt oder verzogert und unregelmassig gespalten, im Gegensatz zu den Lactose negativen *species* der *sub genera* I, II und IV

Gelatine wird von den typischen *species* des *sub genus* I nicht verflussigt, dagegen von den *sub-genus* II-, III- und IV-*species* Die Gelatine

Ferner hat der Verfasser im Jahre 1959 Dr *Ingeburg Bohlck*, welche *S kiel* = 1 2,12 g p — publizierte, gebeten in ihrer Arbeit folgende Satze zu schreiben

„*Kauffmann* ausserte in einer schriftlichen Mitteilung seine Ansicht dass der neue Typ, der sich biochemisch wie *S dublin* verhalte in der selben Weise genetisch mit *S dublin* (1,9 12 gp) zusammenhangen konne wie *S paratyphi A* (1 2,12 a) mit *S sendai* (1 9 12 a 1 5) Zwischen den beiden letztgenannten Typen bestehe neben dem phylo genetischen auch ein kultureller und klinischer Zusammenhang (*Kauffmann* 4) *Kauffmann* ist der Meinung dass sowohl *S paratyphi A* aus *S sendai* als auch der neue Typ aus *S dublin* durch Verlust des O Anti gens 9 entstanden sein konnten Fur die Wahrscheinlichkeit dass es sich um Verlustvarianten handelt, sprechen auch die Vermutungen von *White*, der monophasische Stamme als Verlustvarianten diphasischer Typen ansah

Diese Vermutung des Verfassers wurde spater durch Dr *I Bohlck* bestatigt, da sie im Jahre 1964 mitteilte, dass der *S kiel* Ausscheider unter 10 positiven Befunden die seit 1958 erhoben werden konnten zweimal *S dublin* aufwies Eine Mitteilung hieruber befindet sich im Archiv fur Hygiene und Bakteriologie im Druck [1] Diese Befunde machen es sehr wahrscheinlich dass der Bacillentrager mit *S dublin* infiziert war, unde dass dann spater in seinem Korper die Verlustvariante *S kiel* entstanden ist

ZUSAMMENFASSUNG

In einem Supplement zum *Kauffmann White Schema* werden 33 neue *Salmonella species* und 3 Varianten des *sub genus* I die im Laufe des Jahres 1964 festgestellt wurden mitgeteilt Die neuen *species* der *sub genera* II III und IV sind in einer folgenden Mitteilung enthalten

Bis Ende des Jahres 1964 waren ca 950 *Salmonella species* der *sub genera* I II und IV festgestellt worden Wenn hierzu die Arizona kul turen (*sub genus* III) gerechnet werden kommen wir auf eine Zahl von uber 1000 *Salmonella species*

LITERATUR

Bohlck I Ein neuer Salmonellatyp *S kiel* (1 2 12 g p —) Arch Hyg und Bakter *143* 82 84 1959

Kauffmann F (1) Die Bakteriologie der Salmonella Species (Munksgaard Kopen hagen 1961)

Kauffmann F (2) Das Kauffmann White Schema in Ergebnisse der Mikrobiologie Immunitätsforschung und Experimentellen Therapie *30* 160–216 1957 (Sprin

ann White Schema (VII) Acta path

Z Hyg *1?0* 177 197 1938

Pr blem of Salmonellosis herausgege

en Haag Nederlande 1964

1 (Arch Hyg und Bakter *149* 185–187 1965)

TABELLE 1 (fortgesetzt)

	0	H 1 Phase	H 2 Phase
S vredelust	1 13 23 13	1 z_{28} L	z_{42} z_{42}
S bornheim	1 6 14 25 C	z_{10} z_{10}	1,(2) 7 1
S louwbester	16 16	z z	e,n x e n
S woerden	17 17	c c	z_{39} z_{39}
S shomron	18 18	z_4 z_{32} z_4	— —
S odijk	30 30	a a	z_{39} z_{39}
S.	35 35	z_{29} z_{29}	e,n x e n
S dubrovnik	41 41	z z	1,5 1
S nuernberg	42 42	z z	z_6 z_6
S	48 48	g,m (s) t G	— —
S setubal	60 60	g m t G	z_6 z_6
Species des Sub Genus III			
S arizonae	57 Ar 34	1 Ar 33	z Ar 31
Species des Sub Genus IV			
S argentina	6 7 C	z_{36} z_{36}	— —
S chameleon	16 16	z_4 z_{32} z_4	— —
S seminole	1 40 40	g (p) z_{51} G	— —
S marina	48 48	g (p),z_{51} G	— —
S bockenheim	1 53 53	z_{36} z_{38} z_{36} z_{38}	— —

Die Antigenformeln der *sub genera* II und IV sind in der 1 Linie nach dem originalen in der 2 Linie nach dem vereinfachten K W-Schema angegeben

verflüssigenden *species* des *sub-genus* I bilden eine besondere, biochemische Untergruppe innerhalb des *sub-genus* I

Von den organischen Säuren sei besonders das *Malonat* hervorgehoben, da die *sub-genus* I- und IV-*species* hierin eine negative Reaktion, die *sub-genus* II- und III-*species* aber eine positive Reaktion ergeben In *d-Tartrat* zeigen die meisten *sub-genus* I-*species* eine prompt positive Reaktion, während die *sub-genus* II-, III- und IV-*species* eine negative oder spät und unregelmässig eintretende, positive Reaktion aufweisen

Die *species* der *sub-genera* I, II und III ergeben einen negativen KCN-Test, im Gegensatz zu den typischen Vertretern des *sub-genus* IV, die dadurch ein Bindeglied zu den KCN-positiven *Citrobacter*-Kulturen bilden

In serologischer Hinsicht ist auffallend, dass die bisher bekannten *species* des *sub-genus* IV alle monophasisch sind und nur in der 1 Phase vorliegen Bisher sind nur 3 verschiedene H-Antigen-Komplexe nachgewiesen, nämlich der G-Komplex = $g,(p),z_{51}$, der z_4-Komplex und der z_{36}-Komplex

Alle bisher bekannten *species* des *sub-genus* IV sind in einer Mitteilung des Verfassers *„Das Salmonella Sub-Genus IV“*, die in den Annales Immunologiae Hungaricae als Festschrift für Professor Dr *K Rauss* 1965 erscheint, zusammengestellt

Im Jahre 1964 wurden 19 *species* des *sub-genus* II, 1 *species* des *sub genus* III und 5 *species* des *sub genus* IV festgestellt Ihre Antigen Formeln und Vergärungsreaktionen sind in den Tabellen 1 und 2 angegeben

TABELLE 1

	0	H 1 Phase	H 2 Phase
	Species des Sub Genus II		
S makoma	4 12	a	—
	B	a	—
S kluetjenfelde	4 12	d	e n x
	B	d	e n
S heilbron	6 7	l z_{28}	1 5 z_{42}
	C	L	1 z_{42}
S suederelbe	1 9 12	b	z_{39}
	D	b	z_{39}
S duivenhoks	(9) 46	g m s t	e n x
	D	G	e n
S matroosfontein	3 10	a	e n x
	F	a	e n
S stikland	3 10	m t	e n x
	I	G	e n
S srinagar	11	b	e n x
	11	b	e n

Tabelle 2

+2 = positiv nach 2 Tagen — = negativ nach 14 Tagen ... massig positiv oder negativ

---

Als Ergänzung zur Tabelle seien noch einige Abweichungen aufgeführt

S makoma spaltete prompt Salicin

S kluetjenfelde spaltete ebenfalls prompt Salicin und bildete Indol

S srinagar und *S bornheim* waren Sorbit-negativ

S argentina gab eine negative Reaktion in Ammonium-Citrat

Salicin wurde von *S argentina*, von *S marina* und von *S bockenheim* verzögert, von *S chameleon* und *S seminole* aber prompt gespalten

Alle 5 *species* des *sub-genus* IV waren KCN-positiv

S = 48 g,m (s),t — spaltete Sorbit nach 4 Tagen und war negativ in Ammonium Citrat

Die Arizona Kultur 57 1 z spaltete Lactose prompt

S bockenheim wurde aus den Faeces eines indischen Dornschwanzes und dem Urin einer *Boa constrictor* des Frankfurter Zoo isoliert und von *R Rohde* bestimmt

S argentina = 6 7 z_{36} — wurde 1964 von *Kallings*, Stockholm, aus einem Angehörigen des Schiffes „Argentina" isoliert Diese *species* war bereits 1956 von *L Huber*, Bern, aus einem Falle menschlicher Enteritis gezüchtet worden Der Patient war von den Balearen heimgekehrt Diese Kultur wurde von *Edwards, Kauffmann & Stucki* als „atypical Arizona" beschrieben Es geht hieraus hervor, dass sich unter alten „Arizona" Stämmen nicht nur *sub genus* III-, sondern auch *sub-genus* II- und IV *species* befinden

Da alle typischen *species* des *sub genus* IV KCN-positiv sind, kann man dieses *sub genus* als Bindeglied zwischen *Salmonella* und *Citrobacter* auffassen Ferner wird die Definition des *sub-genus* II durch Ausschluss der „atypischen" Kulturen, die nun das *sub genus* IV bilden, klarer und einheitlicher

Addendum zu „Weitere Salmonella-species des sub genus II (1963)"

1554 = 6,7 b e n,x z_{42} = *S bloemfontein*
1628 = 6 7 z z_{42} = *S oysterbeds*
1636 = 11 g (p),z_{51} — — *S mundsburg* = *sub genus IV*
Aus einem Gecko von Madagaskar
1568 = 1,13 23 g,(m) t — = *S kraaifontein*

TABELLE 2

Biochemisches Verhalten der Sub Genera II, III und IV

	Ara	Dul	Ino	Rha	Xyl	Tre	Gly	H_2S	Cel	d	I	i	Cit	Muk	Mal
Sub Genus II															
S. makoma	+	+	—	+	+	+	++	+	+	×	—	—	$+^{2}$	+	+
S. kluetjenfelde	+	+	—	+	+	+	++	+	+	×	—	—	$+^{3}$	+	+
S. heilbron	+	+	+	+	+	+	++	+	+	×	—	—	$+^{-}$	+	+
S. suederelbe	+	+	—	+	+	+	++	+	+	×	—	—	$+^{-}$	+	+
S. duivenhols	+	+	—	+	+	+	++	+	+	—	—	—	$+^{2}$	+	+
S. matroosfontein	+	+	+	+	+	+	++	+	+	—	—	—	×	+	+
S. stikland	+	+	—	+	+	+	++	+	+	—	—	—	$+^{2}$	$+^{2}$	+
S. srinagar	+	+		+	+	+	++	+	+	×	—	—	$+^{2}$	+	—
S. vredelust	+	+	—	+	+	+	++	+	+	×	—	—	$+^{2}$	+	+
S. bornheim	+		—	+	+	+	++	+	+	×	—	—	+	+	—
S. louw bester	+	+	+	+	+	+	++	+	+	—	—	—	$+^{-}$	$+^{2}$	+
S. woerden	+	+	—	+	+	+	++	+	+	×	—	—	$+^{-}$	+	+
S. shomron	+	—	—	+	+	+	++	+	+	—	—	—	$+^{2}$	$+^{2}$	+
S. odijk	+	—		+	+	+	—	+	+	×	—	—	$+^{3}$	+	+
S. 35 zog e n x —	+	+	—	+	+	+	++	+	+	×	—	—	$+^{2}$	+	+
S. dubrovnik	+	+	—	+	+	+	++c	+	+	×	—	—	$+^{3}$	+	+
S. nuernberg	+	+	—	+	+	+	—	+	+	×	—	—	$+^{2}$	+	+
S. 48 g m (s) t	+	+	—	—	+	+	—	+	+	—	—	—	$+^{4}$	$+^{4}$	—
S. setubal	+	+		+	+	+	++	+	+	×	—	—	$+^{2}$	+	+
Sub Genus III															
S. arizonae 57 i z	+	—	—	+	+	+	++	+	+	—	—	—	$+^{-}$	$+^{2}$	+
Sub Genus IV															
S. argentina	+	—	—	+	+	+	-	+	+	—	—	—	$+^{2}$	—	—
S. chameleon	+	—	—	+	+	+	—	+	+	—	—	—	$+^{2}$	—	—
S. seminole	+	—	—	+	+	+	—	+	+	—	—	—	$+^{2}$	—	—
S. marina	+	—	—	+	+	+	—	+	+	×	—	—	$+^{-}$	—	—
S. bockenheim	+	—	—	+	+	+	—	+	+	×	—	—	$+^{2}$	—	—

+2 = positiv nach 2 Tagen — = negativ nach 14 Tagen [illegible]
ma s g positiv oder negativ

Als Ergänzung zur Tabelle seien noch einige Abweichungen aufgeführt

S. makoma spaltete prompt Salicin

S. kluetjenfelde spaltete ebenfalls prompt Salicin und bildete Indol

S. srinagar und *S. born*

S. argentina gab eine [illegible] *nheim*

Salicin wurde von *S. a* [illegible]

verzögert von *S. chameleon* und *S. seminole* aber prompt gespalten

Alle 5 *species* des *sub genus* IV waren KCN positiv

S = 48 g m (s) t — spaltete Sorbit nach 4 Tagen und war negativ in Ammonium Citrat

Die Arizona Kultur 57 [illegible] z spaltete Lactose prompt

S. bockenheim wurde aus den Faeces eines indischen Dornschwanzes und dem Urin einer *Boa constrictor* des Frankfurter Zoo isoliert und von *R. Rohde* bestimmt

S. argentina = 6 7 z_{36} — wurde 1964 von *Kallings* Stockholm aus einem Angehörigen des Schiffes Argentina isoliert Diese *species* war bereits 1956 von *I. Huber* Bern aus einem Falle menschlicher Enteritis gezüchtet worden Der Patient war von den Balearen heimgekehrt Diese Kultur wurde von *Edwards Kauffmann & Stucki* als atypical Arizona" beschrieben Es geht hieraus hervor dass sich unter alten Arizona Stämmen nicht nur *sub genus* III sondern auch *sub genus* II und IV *species* befinden

Da alle typischen *species* des *sub genus* IV KCN positiv sind kann man dieses *sub genus* als Bindeglied zwischen *Salmonella* und *Citrobacter* auffassen Ferner wird die Definition des *sub genus* II durch Ausschluss der atypischen Kulturen die nun das *sub genus* IV bilden klarer und einheitlicher

Addendum u Weitere Salmonella species des sub genus II (1963)

1554 = 6 7 b e n x z_{1} = *S. bloemfontein*

1628 = 6 7 z z_{1} = *S. oysterbeds*

1636 = 11 g (p),z_{51} — = *S. mundsburg* = *sub genus IV*
Aus einem Gecko von Madagaskar

1568 = 1 13 23 g (m) t — = *S. kraaifontein*

1550 = 16: z_4,z_{53}: — = *S. ochsenzoll* = *sub-genus* IV.
Aus einer erkrankten Python-Schlange eines Zoo
1549 = 30: f,g,p,t: — = *S. slatograd*
1600 = 40: g,p: — = *S. maartendijk* = *sub-genus* III.
1565 = 44: z_4,z_{32}: — = *S. lohbruegge* = *sub-genus* IV.
Aus einer gesunden Python-Schlange eines Zoo
1631 = 47: z_6: 1,6 = Malonat-positiv
1625 = 48: d: 1 = keine neue *species*, da die 2. Phase wahrscheinlich eine R-Phase (1,11) ist.
1607 = 50: g,m,s,t: 1,5 = *S. namib.*
1593 = 50: z_4,z_{23}: — = *S. flint* = *sub-genus* IV. Siehe auch bei *McWhorter, Ball & Ewing*. Internat Bull Bacter. Nomenclat & Taxon. *14*: 59–60, 1964
1573 = 51: z_4,z_{23}: — = *S. harmelen* = *sub-genus* IV.

ZUSAMMENFASSUNG

Es werden die im Jahre 1964 festgestellten 25 *species* der *Salmonella-sub-genera* II, III und IV mitgeteilt.

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Acta path. et microbiol scandinav 64, 373–380, 1965.

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The Royal Dental College, Copenhagen

BACTERIA IN WHIPPLE'S DISEASE

3 Studies in Two Patients of Antibodies in Serum and Cutaneous Hypersensitivity against Some Bacterial Antigens

By

R Dybkær and N Kok

Received 24 i 65

In two patients with typical Whipple's disease cultivation from several duodenal mucosal biopsies yielded many strains of microorganisms (18)—mostly contaminants It was impossible, of course, to identify conclusively any of these strains with the slender rods, about 0 2 × 1 4 µm, seen in electron micrographs from the mucosa Preparations of antigens from some of the microbes and related strains were used in tests for cutaneous hypersensitivity and for *in vitro* studies on circulating antibodies In view of the electron microscopical findings, however, this report will be confined, mainly, to the serological investigations on antigens from small, rod shaped bacteria

MATERIALS AND METHODS

to 1 10 diluti n against undiluted serum

The work of the first author has been supported by grants from *Frøken P A Brandt s Legat*", F L Smidth & Co A S s *Jubilæumsfond* *Nordisk Insulinfond*", and "*Statens almindelige Videnskabsfond*

TABLE 1
The Strains of Bacteria Mentioned in the Text and from Which Antigens Were Prepared

Bacterial strain		Source	
No	Taxonomy	Case No	Biopsy No
1	*Corynebacterium sp*	1	2
2	*Corynebacterium sp*	1	3
3	*Corynebacterium sp*	1	4
4– 8	*Corynebacterium spp*	2	2
9 11	*Corynebacterium spp*	2	3
12	*Streptomyces* like	1	2
13	*Brucellaceae* like	1	3
14	*Brucellaceae*-like	1	5
15	*Brucellaceae* like	2	2
16	*Salmonella sp*	lab , type strain	
17	*Pasteurella multocida*	lab , type strain	
18	*Pasteurella pseudotuberculosis*	lab , type strain	
19	*Bordetella pertussis*	lab , type strain	
20	*Bordetella parapertussis*	lab , type strain	
21	*Brucella abortus*	lab , type strain	
22	*Haemophilus influenzae* type b	lab , type strain	

The strains Nos 1–15 were isolated from material obtained by peroral duodenal biopsies in the two patients, the strains Nos 17–22 were laboratory type strains kindly supplied by *W Frederiksen* M D, Diagnostic Department, Statens Serum institut The biopsy numbering is the same as in an earlier paper (18) The strains numbered 1, 2 and 5 or 6 are identical with Nos 1, 3, and 10, respectively, in the same report

In *agar gel plate precipitation tests ad modum Ouchterlony*[1] the suspension was heated at 60 °C for one hour for the first series of experiments (19 IX 63) ground in a porcelain mortar for the second series (14 XI 63) The antigens were deposited in 4 mm cylindrical holes in a 2 3 mm thick water agar plate (previously stored in an air tight container for several weeks) with serum in a long trench between two rows of holes Several times during the ensuing four weeks (at room temperature) the plates were inspected by dark field illumination

For

60 °C

pensio

saline

each primary wheal and of the wheal and erythema 30–45 minutes, 24 and 48 hours later were measured to the nearest millimetre

The sera were obtained from the two patients mentioned as well as from some controls Serum from a patient with Whipple's disease in Great Britain[2] and from one in the U S A[3] were also run in the agar gel precipitation tests, results from these two sera should be interpreted with reservation because of heavy microbial growth in the samples

1 Materials used were kindly supplied by

3 The serum was kindly supplied by C [illegible] Ashworth M D, Department of Pathology, University of Texas, South Western Medical School, Dallas, Texas, U S A

RESULTS

Agglutination and Ring Precipitation Tests

and a *Brucellaceae*-like strain No 13 are summarized in Table ...

The *Corynebacterium* No 1 agglutinated in two "early" samples of serum diluted 0 0025 (= 1/400), but not in later samples; the strain showed no precipitation, the *Brucellaceae*-like strain No 13 agglutinated spontaneously and gave precipitation in "early" sera, not in "later"

Agar Gel Precipitation Tests

Two "late" sera from the first patient (24 X 62 and 14 V 63), an "early" (11 VI 63) and a "late" (16 IX 63) serum from the second patient, the donated sera from two foreign patients with Whipple's disease, and serum from a normal person were used

The antigens derived from the *Streptomyces*-like strain No 12, eleven different corynebacteria (Nos 1–11), and three *Brucellaceae-like strains* (Nos 13-15), the brucellaceae were tested both as heated and ground antigens

None of the antigens elicited precipitation lines with any of the sera

Intracutaneous Injections

Tests for cutaneous hypersensitivity of the humoral (immediate, I-) and cellular (delayed, D-) types were made on the dates given in Table 2 In case 1 the following strains were tested in addition to those listed the *Streptomyces*-like strain No 12, *Salmonella* No 16, and corynebacteria Nos 2 and 9 11 in case 2 *Corynebacterium* No 2 All these tests gave zero results, *i e* less than the control injection or a wheal less than 8 mm in diameter

Table 2 shows that *the first patient* did not react to *Corynebacteria*, not even to strain No 1 which early samples of his serum agglutinated Among the laboratory *strains of identified Brucellaceae* the two species of *Bordetella* (Nos 19 and 20) and the *Haemophilus influenzae* strain No 22 gave zero reactions, whereas the *Brucella abortus* strain No 21 elicited definite I- and D-reactions as "undiluted" antigens and the two species of *Pasteurella* (Nos 17 and 18) D-reactions in the same concentration All the three *Brucellaceae*-like strains Nos 13–15 yielded positive I- and or D-reactions at some time or other, and, notably, the strain No 15 isolated from patient No 2

The second patient gave D-type reactions to several strains of *Corynebacterium* (Nos 3 8) and definite reactions against his own *Brucellaceae* like strain No 15 and the two strains Nos 13 and 14 from case 1

In three normal, male persons the *Brucellaceae*-like strains Nos 13 and 14 were tested as "undiluted" antigens at a time (21 V 63) when the

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12	*Streptomyces* like	1	2
13	*Brucellaceae* like	1	3
14	*Brucellaceae* like	1	5
15	*Brucellaceae* like	2	2
16	*Salmonella sp*	lab type strain	
17	*Pasteurella multocida*	lab type strain	
18	*Pasteurella pseudotuberculosis*	lab type strain	
19	*Bordetella pertussis*	lab type strain	
20	*Bordetella parapertussis*	lab type strain	
21	*Brucella abortus*	lab type strain	
22	*Haemophilus influenzae* type b	lab type strain	

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1 *Materials used* were kindly supplied by
cal
2
Edg
3 The serum was Department of Pathology University of Texas South Western Medical School Dallas Texas U S A

...same antigens gave weak D-reactions in the first patient (17 V 63)
... D-reaction, and

DISCUSSION

The elucidation of the pathogenesis in Whipple's disease is difficult because blood cultures have proven sterile and isolation of the bacteria seen in the jejunal lamina propria is complicated by contamination from the luminal microflora. The aetiological significance of isolated microbial strains from jejunal biopsies can hardly be determined by classical methods; indirect criteria will have to suffice.

Earlier Reports

Results of routine serological investigations have been reported repeatedly in the literature.

[illegible]
many zero results have been reported to incriminate tubercle bacilli (4, 13, 20, 21, 24).

Several authors have found antibodies in serum against *Streptococci* (9, 15, 23, present case 1), but this is of scant value as the microorganisms of interest seem to be rod-shaped.

Agglutination reactions (Widal) against *Salmonellae* are zero (1, 3, 9, 11, 14, 20, 21, 23), and an anti H titre of 50 in the present case 1 was explained by an earlier vaccination. *Brucellae* have never yielded positive agglutination reactions (1, 11, 14, 16, 20, 21, present case 1), and neither have *Shigellae* (11, 20). Different serological reactions with several *rickettsial* antigens also gave zero results (3, 16, 20).

Cutaneous hypersensitivity could not be demonstrated by *Puite et al* (20) with commercial antigens from *Brucellae, Histoplasmata,* and *Lymphogranuloma venereum*. *Nocardia pulmonalis* isolated by *Kojecky et al* (17) from a jejunal biopsy in a patient with Whipple's disease elicited a late erythema by intracutaneous injection in the patient and in one of ten normal persons; these bacteria were much larger than those in the electron micrographs.

It may be concluded that no earlier reports have revealed pertinent antibodies in patients with Whipple's disease.

Present Investigation

The bacterial antigens produced from bioptical strains for the present investigations suffer from three defects which could not be avoided. Firstly, the primary isolation of each *Brucellaceae*-like strain was difficult and required a long time, and consequently sufficient amounts

TABLE 2

Results from Two Patients with Whipple's Disease Agglutination and Ring Precipitation Tests on Serum as well as Cutaneous Hypersensitivity Reactions

Bacterial strain				Date of sampling																
				Case 1															Case 2	
			Day Month Year	04 05 62	11 05 62	28 05 62	29 05 62	29 06 62	17 07 62	19 07 62	07 09 62	21 09 62	26 09 62	09 10 62	31 01 63	17 05 63	22 11 63	27 11 63	12 09 63	16 09 63
No	Taxonomy	Source	Dilution of antigen			10^{-2}	10^{-1}		10^{-2}	10^{0}		10^{0}	10^{-2}			10^{0}	10^{-1}	10^{0}	10^{-2}	10^{0}
1	*Corynebacterium sp*	1–2		A p	A p	i d	i d		i d	i d	a p	i d		a p						
3	*Corynebacterium sp*	1–4							i d	i d		i d								
4 8	*Corynebacterium spp*	2–2																	i D	i** D**
13	*Brucellaceae* like	1–3																	i D	i d
14	*Brucellaceae* like	1 5			(A)P			P	i*d*	I*d*	(A)p	I d	i d	(A)p	p	i D	i d	I D	i d	I D
15	*Brucellaceae* like	2 2										i d	i d			i D			i d	i D
—	—	—															I D	I D	i d	i D
17	*Pasteurella multocida*	lab type strain										i D	i d							
18	*Pasteurella pseudotuberculosis*	lab, type strain										i D	i d							
19	*Bordetella pertussis*	lab, type strain										i d								
20	*Bordetella parapertussis*	lab, type strain										i d								
21	*Brucella abortus*	lab type strain										I D	i d							
22	*Haemophilus influenzae* type b	lab, type strain										i d								

The strain numbering refers to Table 1 —The sera were obtained on the dates indicated For preparation of antigens, serological techniques and intracutaneous testing, cf text —The two digits given under "Source" refer to the case number and biopsy serial number, respectively —Abbreviations a = no agglutination, A = agglutination in dilution of serum 0 0025, (A) = spontaneous agglutination, d = delayed cutaneous reaction with induration <8 mm in diameter, D = induration ≧8 mm, but <10 mm, **D** = induration ≧ 10 mm, i = immediate cutaneous reaction with wheal <8 mm in diameter, I = wheal ≧8 mm, but <10 mm **I** = wheal ≧ 10 mm, p = no ring precipitation P = ring precipitation * This antigen was considerably more dilute than others employed on the same date ring precipitation, P = ring precipitation

* This antigen was considerably more dilute than others employed on the same date ** Antigen diluted to 10 1

role in Whipple's disease of small, rod shaped, Gram negative bacteria, possibly of the family *Brucellaceae*

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of pure antigens, unfortunately, were not obtained until the patients had been treated for some time, and the symptoms had subsided. Secondly, the strains may have lost important antigenic characteristics during the necessary, repeated subcultivations. Thirdly, the possible identity with the mucosal microbes cannot be proved. The microbes seen in electron micrographs and by light microscopical histology were interpreted as Gram-negative slender rods (18), but this is at variance with findings by other investigators who report the microorganisms to be Gram-positive (2, 5, 7, 8, 10, 16, 17, 19, 22, 25).

The different tests (cf. Table 2) have shown *agglutinins* against the *Corynebacterium* No. 1 and *precipitins* against a *Brucellaceae*-like organism (No. 13) in "early" sera from the first patient, both strains were isolated from "early" jejunal biopsies.

The presence of *cellular antibodies* against small rod-shaped bacteria seems revealed by cutaneous hypersensitivity in both patients. The first did not respond to strains of genus *Corynebacterium*, but reacted with I- and/or D-type lesions against two strains of *Brucellaceae*-like organisms isolated from his own biopsies and against one strain from the other patient. The second patient reacted, mostly with D-type lesions, against all three of the *Brucellaceae*-like strains (Nos. 13–15) and against several corynebacteria (Nos. 3–8).

The antigens which elicited cutaneous reactions in the two patients were not confined to the strains from the jejunal biopsies as known members of the family *Brucellaceae*, *i. e.* two pasteurellae and *Brucella abortus*, yielded similar results. The reactions may not be regarded as unique in patients with Whipple's disease, however, as two out of three normal persons also responded.

Additional evidence might be obtained from fresh jejunal bioptical tissue in an untreated patient by fluorescent antibody techniques.

CONCLUSIONS AND SUMMARY

Several jejunal biopsies from two patients with Whipple's disease yielded growth from many different microbial strains, but owing to electron microscopical findings the interest centered on slender rod-shaped bacteria. The many species of *Corynebacterium* did not resemble the *C. anaerobium* which *Caroli et al.* claimed to be the cause of the disease (5, 6). The three strains of a *Brucellaceae*-like organism seemed compatible with the rods in the electron micrographs.

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The results may lend some additional evidence for a pathogenetic

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Acta path. et microbiol. scandinav. 64, 381–386, 1965

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GROWTH OF *BACTERIUM ANITRATUM* (B5W) WITH NITRATE OR NITRITE AS NITROGEN SOURCE

By

K. Jyssum and P. E. Joner

Received 11.1.65

The organism here termed *Bacterium anitratum* belongs to a group of Gram negative bacilli which are strictly aerobic and grow well on simple media. This microbe was originally provided with the symbol BaW by *Stuart & van Stratum* (11), but it is probably identic to the "P bacillus" described by *Parr & Galbraith* (9). The name *Bacterium anitratum* was coined by *Schaub & Hauber* in 1948 (10). Since that time several proposals as to its nomenclature have been forwarded. In 1961 *Lautrop* (5) suggested that the *B. anitratum* should be transferred to the genus *Cytophaga* since gliding motility, a characteristic of Myxobacteriales, was observed. An elaborate report concerning the characteristics of the organism in question has been published by *Mannheim & Stenzel* (6).

During an examination of several species with regard to their inorganic nitrogen metabolism some observations were made with *B. anitratum* which pointed to the existence of a metabolism very much different from that expected from previous reports (6, 3). Systematic studies were accordingly started.

In the present communication the question has been asked whether *B. anitratum* may not turn out to be genetically equipped to assimilate nitrate or nitrite, although no phenotypic expression of this property may be demonstrated in the experimental systems previously used (10, 6, 3).

MATERIALS AND METHODS

Bacterial strains. The microbes employed have been used in previous work in this laboratory (3). The main test microbe was the strain H-13. This strain was No. 8 of 13 strains of *B. anitratum* obtained from Dr. *Lautrop*, The State Serum Institute, Copenhagen. The strains have been the subject of extensive examination so as to verify their identity (3).

Me[illegible]

[illegible] source were
[illegible] mg per 600 ml
[illegible])$_2SO_4$. Nitrate
[illegible] Agar (Difco)

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GROWTH OF *BACTERIUM ANITRATUM* (B5W) WITH NITRATE OR NITRITE AS NITROGEN SOURCE

By

K Jyssum and P L Joner

Received 11 i 65

The organism here termed *Bacterium anitratum* belongs to a group of Gram negative bacilli which are strictly aerobic and grow well on simple media This microbe was originally provided with the symbol B5W by *Stuart & van Stratum* (11), but it is probably identic to the "P bacillus" described by *Parr & Galbraith* (9) The name *Bacterium anitratum* was coined by *Schaub & Hauber* in 1948 (10) Since that time several proposals as to its nomenclature have been forwarded In 1961 *Lautrop* (5) suggested that the *B anitratum* should be transferred to the genus *Cytophaga* since gliding motility, a characteristic of Myxobacteriales was observed An elaborate report concerning the characteristics of the organism in question has been published by *Mannheim & Stenzel* (6)

During an examination of several species with regard to their inorganic nitrogen metabolism some observations were made with *B anitratum* which pointed to the existence of a metabolism very much different from that expected from previous reports (6, 3) Systematic studies were accordingly started

In the present communication the question has been asked whether *B anitratum* may not turn out to be genetically equipped to assimilate nitrate or nitrite, although no phenotypic expression of this property may be demonstrated in the experimental systems previously used (10, 6 3)

MATERIALS AND METHODS

[illegible] *used in previous work in this* [illegible] n H 13 This strain was No 8 [illegible] *op* The State Serum Institute, [illegible] xtensive examination so as to verify their identity (3)

Media. Heart infusion broth (HIB) was used as liquid complete medium Solid complete media were either blood agar plates or HIB agar plates A minimal medium with ammonium salt as the nitrogen source was prepared as described by *Davis &* [illegible]

[illegible] were added from sterile filtered solutions recently prepared Agar (Difco)

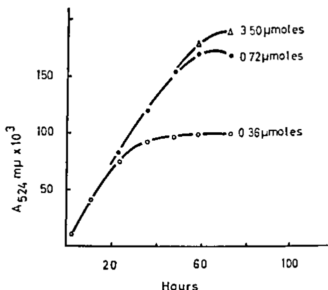


Fig 1

Growth response of *B anitratum* to nitrate Various quantities of KNO_3 (μmoles per ml) used as sole nitrogen source

was used for the solid minimal media Glucose in a concentration of 0 1 per cent in the liquid and 0 5 per cent in the solid media was used as the energy and carbon source along with the sodium citrate of the basal medium (1) Citrate is metabolized by *B anitratum* (6) The preparation of the media was essentially as described in previous communications (2)

Analytical procedures Nitrate was measured by the brucine method described by *Noll* (8) Nitrite was determined by the method of *Medina & Nicholas* (7) and protein analysed by a biuret method (4)

Spectrophotometry was performed either with a Hilger & Watts Uvispec spectro photometer or in a Beckman DB spectrophotometer In both instruments analyses were performed with 1 cm light path Growth in fluid cultures was followed by measurements at 524 mμ

RESULTS

The nitrogen requirements of *B anitratum* (wild type) is satisfied by $(NH_4)_2SO_4$ in the minimal medium of *Davis & Mingioli* (1) Nitrate or nitrite may replace the ammonium salt as the nitrogen source This was first shown by growth on the surface of agar media where all nitrogen was supplied either as KNO_3 or as $NaNO_2$

TABLE 1

Removal of KNO_3 when used as Sole Nitrogen Source in Cultures of B anitratum

Expt	KNO_3 added to 5 ml (μmoles)	KNO_3 removed from 5 ml after 72 h (μmoles)	Increased absorbancy of culture after 72 h (A_{524} mμ × 10^3)
1	36 1	2 6	168
2	18 5	3 3	162
3	7 2	3 6	151
4	3 6	3 5	157
5	1 8	1 8	96
6	0	0	0

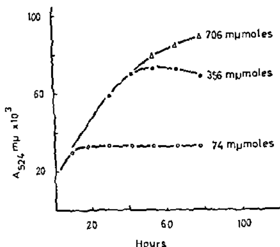


Fig 2
Growth response of *B anitratum* to nitrite Various quantities of $NaNO_2$ (mμmoles per ml) used as sole nitrogen source

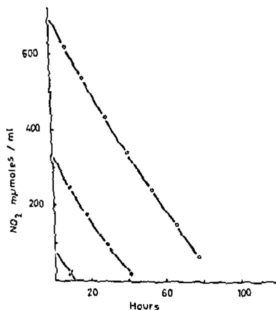


Fig 3
Disappearance f nitrite during growth of *B anitratum* $NaNO_2$ provided as sole nitrogen source in three cultures.

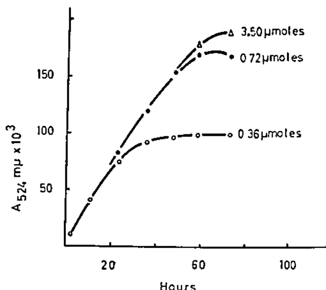


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to the nitrogen recovered from the bacterial mass ... limited by the quantity of nitrate or nitrite added In the absence of an added nitrogen source no growth occurs On the basis of this evidence it is assumed that nitrate or nitrite is indeed assimilated and used as the nitrogen source in *B anitratum*

It may be argued that the faculty to grow with nitrate or nitrite as the nitrogen source is a property found only in some strains However, as seven strains generally recognized as *B anitratum* (B5W) have the property it may be tentatively accepted as a common feature

Since *B anitratum* can be adapted to growth with either nitrate or nitrite as nitrogen source it must possess the genetic equipment necessary to synthesize enzymes which constitute a metabolic pathway leading from NO_3 to amino acids With a view to the consistently negative attempts by many workers to demonstrate an activity corresponding to such a pathway (10 12, 6 3) it may be assumed that this genetic property is only phenotypically expressed under certain defined conditions Therefore it becomes pertinent to search for mechanisms of enzyme regulation which may explain previous negative results

SUMMARY

Bacterium anitratum (B5W) has been examined with regard to its requirements to inorganic nitrogen Six strains could grow on minimal media with either nitrate or nitrite as the sole nitrogen source One strain which turned out to be an auxotroph with growth response to leucine grew well on the same media upon the addition of the growth factor During growth the nitrate and the nitrite added was removed from the medium in quantities corresponding to the nitrogen content of the bacteria synthesized The growth could be limited by the quantity of nitrate or nitrite added It is assumed that nitrate or nitrite may indeed be assimilated and used as the nitrogen source in *B anitratum* (B5W)

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TABLE 2

Removal of $NaNO_2$ *when Used as Sole Nitrogen Source in Cultures of* B *anitratum*

Expt	NaNO added to 5 ml (μmoles)	NaNO removed from 5 ml after 72 h (μmoles)	Increase in absorbancy of culture after 72 h ($A_{mμ}$ × 10^3)
1	17 30	2 50	57
2	8 57	4 72	105
3	3 53	2 77	74
4	1 78	1 78	60
5	0 37	0 37	28
6	0	0	0

Also in fluid cultures nitrate or nitrite may provide *B anitratum* with the nitrogen required for growth Growth with KNO_3 as the nitrogen source is shown in Fig 1 That nitrate is actually removed from the medium during growth could be demonstrated in the type of experiment recorded in Table 1 The quantities removed from the medium during these experiments were found to correspond well with the nitrogen which was recovered from the bacteria synthesized

The growth of *B anitratum* with $NaNO_2$ as the nitrogen source has been shown in Fig 2 The successive removal of nitrite from the medium during growth is demonstrated in Fig 3 This has been further emphasized by some data recorded in Table 2 When the data from Table 2 are compared with those of Table 1 it should be noted that the method used for analysis of nitrite (7) is far more exact than that used for the measurement of nitrate (8) With a view to this methodologic difference the data seem to be in accordance

The experiments show that nitrite is more toxic than nitrate Concentrations of $NaNO_2$ above 1 7 μmoles per ml depress the growth rate to an increasing extent KNO_3 on the other hand, may be added in concentrations above 7 μmoles per ml without a significant reduction of the growth rate

Seven strains generally accepted as *B anitratum* were thoroughly examined Six of the strains could be adapted to growth with nitrate as the sole source of nitrogen These were the strains H-1, H-13, H-14 H 23, H 29, and H-34 (3) One strain (H-26) which failed to grow under the same conditions turned out to be an auxotroph which required leucine for growth The strain grew well on the basal medium with KNO_3 as nitrogen source when supplemented with leucine Also in the case of this strain NO_3 is removed from the medium

DISCUSSION

B anitratum (B5W) may easily be adapted to growth on the surface of agar media or in fluid cultures in which nitrate or nitrite is the only source of nitrogen added

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By

K Jyssum and P E Joner

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Bacterium anitratum (B5W) may be adapted to growth on minimal media in which nitrate or nitrite is the only nitrogen source added (6) If the nitrate or nitrite which is removed from these media during growth is assimilated, the cells must possess enzymes which constitute a metabolic pathway from NO_3 to amino acids An assumed absence of such a pathway has been considered of sufficient taxonomic value to give the bacterium its epithet hence, it becomes pertinent to search for mechanisms of enzyme regulation which may explain previous negative results (19 22 10 3)

The adaptive nature of the assimilatory nitrate reductases seems to be clearly established in *Neurospora crassa* and in higher plants (13) Whether the regulation is by repression or by induction, however, is far from clear Most of the evidence is based on the demonstration that cell free preparations of tissues exposed to ammonium salts or amino acids show little or no activity in comparison to the high activity in nitrate grown material *Morton* (12) observed an inhibiting effect of ammonia on the reductase in certain fungi and ascribed this to an adverse effect on the formation and stability of the enzyme This observation may point to the existence of repression in certain systems

Although most of the evidence points to an inducible nature of the respiratory nitrate reductases in microorganisms (13), there are some reports which fail to support this view *Straughn* (21) was unable to obtain an enhanced nitrate reductase activity by the addition of nitrate to an *E coli* strain grown on peptone broth

MATERIALS AND METHODS

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MATERIALS AND METHODS

The methodology and experimental manipulations were analogous to those previously described (6, 7). The main test microbe was the *B. anitratum* strain H 13.

L-glutamic dehydrogenase and aspartase activities have been measured according to the procedures previously used (4, 5). L-alanine dehydrogenase was measured as described by *Wiame, Piérard & Ramos* (24). Hydroxylamine was determined accord-

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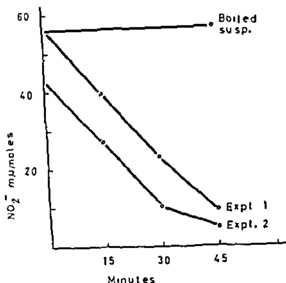


Fig 2

Reduction of nitrite by a suspension of *B anitratum*
Disappearance of nitrite in the absence of $CaCl_2$

of the reaction may be taken as representative for the activity of a reaction NO_3 to NO_2. Over a period of five minutes a suspension of *B anitratum* produced 9 mμmoles nitrite from nitrate while the same suspension diluted 1 2 produced 4 2 mμmoles

B anitratum may also reduce nitrite A removal of NO_2 from solutions was observed when 0 1 μmole $NaNO_2$ was mixed with 1 ml of a suspension of bacteria in "minimal salt solution" Under the conditions of the experiment of Fig 2 the disappearance of nitrite takes place linearly with time during the first 15 to 30 minutes It is accordingly assumed that the initial rate of nitrite disappearance may be accepted, at least provisionally as a measure for this activity The reaction product of the nitrite reduction has not been identified A synthesis of hydroxylamine the ammonium ion as well as nitrate has been searched for but with negative results

The two reactions described require the addition of TCA intermediates in the way it has been shown in Table 1 for the NO_3 reductase Since the activities refer to intact cells the differences observed could be on the penetration of the substrates Therefore, the TCA intermediate to be used in the reaction should also be present in the medium in which the cells are grown Citrate is known to be easily metabolized by *B anitratum* (10) and was accordingly chosen for the main test system

The method used for measurement of nitrite (11) permits the determination in mμmole quantities, and is far more exact than the

ing to the procedure described by *Roussos et al* (17) which involves the oxidation of hydroxylamine by iodine to nitrite Ammonia was determined by the Conway microdiffusion technique in conjunction with the phenol hypochlorite test for ammonia according to the procedure of *Russel* (18)

RESULTS

Reduction of Nitrate and Nitrite.

In order to study a regulation of the postulated metabolic pathway leading from NO_3^- to amino acids some of the intermediate reactions had to be identified. Furthermore, quantitative systems had to be established in which the activities corresponding to these reactions could be compared

A reaction leading from NO_3^- to NO_2^- was first considered Suspensions of intact cells were prepared from cultures of *B anitratum* grown on the surface of basal agar plates with KNO_3 as the only added source of nitrogen. The cells were harvested, washed with saline and immediately suspended in "basal salt solution" This solution had the following composition · K_2HPO_4 40 mM, KH_2PO_4 22 mM, $Na_3Citrate$ 1.44 mM; $MgSO_4$ 0 4 mM, Na_2SO_4 0 7 mM. The density of an actual suspension was adjusted according to readings of the absorbancy One ml of such a suspension was supplied with 10 μmoles $CaCl_2$ and 1 μmole of the substrate KNO_3 From Fig 1 is seen that the synthesis of nitrite is approximately linear during the first 30 minutes of incubation

It is clear that under the conditions of the assay only the initial rate

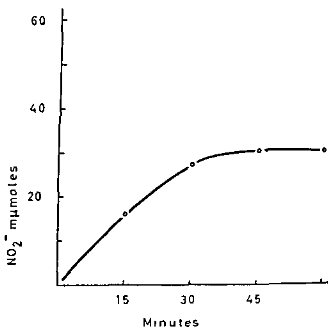


Fig 1

Reduction of nitrate to nitrite by a suspension of *B anitratum* Nitrite accumulation in the presence of $CaCl_2$

reduction in cell free preparations from *B. anitratum* grown on NO_3 corresponding to the activities found in extracts from *Neurospora crassa*, *E. coli* and *Pseudomonas aeruginosa*. So far these attempts have been altogether negative, although control experiments with the previously described systems (13) have been positive.

Repression of the Biosynthesis of the Nitrate Reducing System

Experiments were performed to define the conditions under which the genetic determinants of the nitrite reducing reactions in *B. anitratum* are phenotypically expressed.

TABLE 2

Influence of the Nitrogen Source on the NO_3 and NO_2 Reduction in Suspensions of B. anitratum

Expt	Nitrogen source during growth	Activity measured	A_{540} mμ × 10^3 per 30 min*	Per cent activity
1	NO_3	NO_3 reduction	353	96.5
2	NO_3	NO_3 reduction	366	100
3	NO_2	NO_3 reduction	0	0
4	NH_4	NO_3 reduction	25	6.8
5	NO_3	NO_2 reduction	463	99.5
6	NO_2	NO_2 reduction	465	100
7	NO_2	NO_2 reduction	433	93.3
8	NH_4	NO_2 reduction	71	15.2
9	NH_4	NO_2 reduction	157	33.7
10	casamino acids	NO_3 reduction	0	0
11	casamino acids	NO_2 reduction	0	0

* Measured after addition of NO_2 reagents of *Medina & Nicholas* (11) in suspensions adjusted to the same optical density.

Activities were measured in suspensions of cells grown with NO_3, NO_2 or NH_4 as the nitrogen source. The data given refer to suspensions which were adjusted to the same absorbancy (Dilution 1 : 5 gives A_{540} mμ = 0.320). The unit was arbitrarily chosen. From Table 2 is seen that the activity of the reaction NO_3 to NO_2 is high in suspensions grown on NO_3 but absent in suspensions grown on NO_2. In suspensions grown on NH_4 the activity is fairly low, but it is still present. Furthermore the data of Table 2 show that a synthesis of the enzyme responsible for the NO_3 reduction is repressed by casamino acids. Suspensions from bacteria grown in the medium generally used for the demonstration of NO_3 reduction (10) are also devoid of activity.

This evidence points to a multi enzyme system regulated by repression rather than by induction. The NO_3 reductase is apparently repressed by NO_2, while both reductases are repressed in the presence of casamino acids. The substance responsible for this repression does not seem to be NH_4^+.

Table 3 reports experiments performed to identify the repressors

method available for the measurement of nitrate (15) Also, it has been found that keto-acids strongly interfere with the latter method due to a reaction with brucine (*Joner & Jyssum*, unpublished results) Therefore, it became desirable to find some substance which could selectively inhibit the NO_2^- reduction. Most chemicals affected both reactions in the same way Inhibition was found with CN , As^{3+}, Hg^{2+}, Cd^{2+}, Fe^{2+}, Co^{2+}, while no inhibition could be demonstrated with F , As^{5+}, Mn^{2+}, Li^{+}, Zn^{2+}, NO_3^- However, Ca^{2+} was found to inhibit the NO_2 reduction approximately 30 per cent without a reduction in the rate of nitrate disappearance Presumably, as the result of the inhibition of the nitrite reduction nitrite was accumulated As a consequence of this observation the NO_3 reduction was measured as described above From Fig 1 is seen that the reaction rate levels off after the first 30 minutes The reason may be an inhibition by the reaction product nitrite, or that no more electrons are available Other reasons could be that a steady state has been reached between the production of nitrite and a further reduction, or that some metabolite from nitrite has been formed which exerts a feed back inhibition on the NO_3 reduction

TABLE 1

Effect of TCA Intermediates on the NO_3 and NO_2 Reduction in Suspensions of B anitratum

Substance tested	Per cent activity*
None	0
Citrate	100
Isocitrate	12
α oxoglutarate	7
Succinate	75
Fumarate	100
Malate	40
Pyruvate	77
Oxalacetate	95
Glucose	0

* Measured as A_{540} mμ per 30 minutes after addition of the NO_2^- reagents of *Medina & Nicholas* (11) in suspensions adjusted to the same optical density The system is described in the text

The systems described above are quite complex A more detailed analysis, however, calls for soluble preparations or subcellular fragments of *B anitratum* with similar activity When a suspension in "minimal salts" was treated with 50 μl toluene per 5 ml for 30 minutes at 37° the two reactions were completely stopped although the control suspensions maintained full activity when similarly treated without toluene A treatment with ultrasonic waves for 2½ minutes also stopped the reactions Ultrasonic treatment was also performed in two "stabilizing fluids" with the same result (Fluid 1 KCl 0 15 M, $MgCl_2$ 0 001 M, Mannose 0 25 M, ATP 0 001 M, Tris pH 8 0 005 M Fluid 2 Tris pH 8 0 05 M, sucrose 0 25 M)

Many attempts have been made to demonstrate a nitrate or nitrite

hibition observed in the presence of the ammonium ion is in distinct contrast to the lack of effect from this chemical on the enzymic synthesis Again, glutamic acid is without significant effect

TABLE 4

Inhibition of the NO_3 Reducing Activity of B anitratum Suspensions by Amino Acids and the Ammonium Ion

Substance tested as inhibitor in the assay system	Percent activity in presence of the inhibitor§
none	100
glu	94
pro	91
orn	94
cit	100
arg	68
asp	33
aspargine	13
thr	64
ile	88
met	69
lys	98
ala	80
val	78
leu	88
ser	87
gly	88
cys	23
his	71
phe	90
tyr	78
try	72
β ala	92
NH_4Cl	7
$(NH_4)_2SO_4$	6

2 µmoles of each substance added to the test system

§ Measured as A_{540} mµ per 30 minutes according to the technique described in the text

L Glutamic Dehydrogenase and Aspartase Activities

It is generally assumed that NO_3^- and NO_2^- are reduced to NH_4^+ before incorporation into amino acids (13) The enzyme reactions which are most likely to be responsible for an incorporation had accordingly to be considered

From Table 5 is seen that *B anitratum* possesses a NADP catalysed L-glutamic dehydrogenase which is active in cells grown on blood agar and on minimal medium with NH_4 as the nitrogen source The activity, however is absent from nitrate grown cells as well as from cells grown with L glutamic acid as the only nitrogen source The activity of the L glutamic dehydrogenase per minute per mg protein is of the same dimension as that found in *Neisseria meningitidis* (5) A NAD catalysed L glutamic dehydrogenase activity has also been searched for but with no positive results

B anitratum also exhibits an aspartase activity This activity does

present in casamino acids. Clearly, the amino acids are highly heterogeneous with regard to their effect on the reductive system in question. The repressor effect of a few substances is pronounced. These are aspartic acid, aspargine, arginine, alanine and histidine. Threonine should perhaps also be included in this group since growth is completely stopped in the presence of this amino acid. It is equally clear that no repression is obtained with the ammonium ion or with glutamic acid. Some amino acids stimulate the synthesis of the enzymes responsible for nitrate reduction. This also holds for some keto acids, particularly α-oxoglutaric and oxalacetic acid.

TABLE 3

Repression of the NO_3^- Reducing System of B. anitratum *by Amino Acids*

Substance tested as repressor in NO_3^- culture*	Per cent activity in the resulting suspension§
none	100
glu	100
hydroxypro	100
pro	198
orn	92
cit	136
arg	15
asp	33
aspargine	5
thr	no growth
ile	61
met	77
lys	100
ala	20
val	57
leu	80
ser	57
gly	100
cys	60
his	11
phe	150
tyr	182
try	190
β ala	33
NH_4Cl	100
$(NH_4)_2SO_4$	100

* 50 mg of each substance was added to 100 ml NO_3^- medium.
§ Measured as A_{540} mμ per 30 minutes according to the technique described in the text.

Inhibition of the Activity of the Nitrate Reducing System

In the household of the individual cell the mechanisms regulating the activity of an enzyme reaction must be considered equally important as those regulating the biosynthesis. Search was accordingly started for some mechanism which might indicate an activity regulation by feedback.

In Table 4 has been shown that the reduction of NO_3^- to NO_2^- is effectively inhibited by aspartic acid, aspargine and cysteine. The in-

hibition observed in the presence of the ammonium ion is in distinct contrast to the lack of effect from this chemical on the enzyme synthesis. Again, glutamic acid is without significant effect.

TABLE 4

Inhibition of the NO_3 Reducing Activity of B. anitratum Suspensions by Amino Acids and the Ammonium Ion

Substance tested as inhibitor in the assay system*	Per cent activity in presence of the inhibitor §
none	100
glu	94
pro	91
orn	94
cit	100
arg	68
asp	39
aspargine	13
thr	64
ile	88
met	69
lys	98
ala	86
val	78
leu	88
ser	87
gly	88
cys	29
his	71
phe	90
tyr	78
try	72
β ala	92
NH_4Cl	7
$(NH_4)_2SO_4$	6

* 10 µmoles of each substance added to the test system.
§ Measured as A_{520} mµ per 30 minutes according to the technique described in the text.

L-Glutamic Dehydrogenase and Aspartase Activities

It is generally assumed that NO_3 and NO_2 are reduced to NH_4^+ before incorporation into amino acids (13). The enzyme reactions which are most likely to be responsible for an incorporation had accordingly to be considered.

From Table 5 it is seen that B. an...

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... dehydrogenase activity has also been searched for but with no positive results.

B. anitratum also exhibits an aspartase activity. This activity does

not seem to be influenced by the nitrogen source It is remarkable that the activity in *B anitratum* per mg protein is very much lower than that found in *E coli*, and not increased as the result of an adaption to growth on NO_3

TABLE 5

Measurements of Activities Corresponding to L glutamic Dehydrogenase L alanine Dehydrogenase and Aspartase

Strain	Source of extract	L-glutamic dehydrogenase A_{340} mμ × 10^3	L alanine dehydrogenase L alanine μg	Aspartase NH_3 mμg
B anitratum	Blood agar	27	0	22
B anitratum	NH_4^+ as N	31	0	23
B anitratum	NO_3 as N	0	0	26
B anitratum	glu as N	0	0	–
B anitratum	asp as N		0	25
N meningitidis	Blood agar	36	0	–
B subtilis	Blood agar		3 5	–
E coli K12	Blood agar	–	0	510

All activities are calculated per minute per mg of protein

No L-alanine dehydrogenase activity could be found in *B anitratum* In the control system from *Bacillus subtilis* this activity was easily demonstrated by measurements in both directions The data included in Table 5 were obtained by measurements of NADH oxidation In the same extract a measurement with NAD reduction gave the activity 1 2 μg L-alanine per minute per mg protein

DISCUSSION

The NO_3 reducing system in *B anitratum* differs from those described from the microbes *N crassa, E coli* and *P aeruginosa* (13) The NO_3-reductase *per se* appears to be of a special kind since it has not been possible to demonstrate activity in soluble preparations or in subcellular fractions One explanation could be that the reaction is dependent upon an intact cell surface in *B anitratum* Another explanation, however, is that the enzyme is extremely susceptible to oxidation A soluble nitrate reductase with extreme lability has been found in extracts from *Rhizobium japonicum* (8) NADH and succinate, which were effective electron donors for particulate enzyme preparations, were completely ineffective donors with the soluble preparations Reduced benzyl or methyl-viologen were the only compounds that functioned with the soluble enzyme Another unique nitrate reductase has been found in *Aerobacter aerogenes* (16) in which molecular hydrogen served as the electron source for nitrate reduction Benzyl-viologen had to be present to establish a chemical coupling between hydrogenase and nitrate reductase It remains to be found out whether the nitrate

reductase in *B. anitratum* may be similar to those found in *Bacillus stearothermophilus* (8) or *Aerobacter aerogenes* (16).

The mechanisms regulating the nitrate-reducing systems are apparently not analogous to those previously reported in other micro-organisms. The evidence is taken to indicate that the biosynthesis of the system is regulated by repression in *B. anitratum*.

Aspartic acid and alanine seem to occupy a central position among the repressors. Three of the effective repressors, asparagine, threonine and iso-leucine are biosynthetically closely related to aspartic acid. The three other repressors, arginine, histidine and β-alanine include [illegible]

ribophosphate from aspartate, PP-ribose-P, N-formyltetrahydrofolic acid and NH_4^+ (9). Histidine may also be thought to interfere with the aspartic acid metabolism as the result of its catabolism. Bacteria metabolize histidine by several routes with some species differences. The main products from the catabolism seem to be formate, glutamate and aspartate. Although the usual pathway seems to be that leading to glutamic acid (14), the pathway leading to aspartic acid and formate is apparently of importance in some species (23). From studies with *E. coli* Shive & Macow (20) concluded that aspartic acid is a precursor of β-alanine, and *Billen & Lichstein* (1) demonstrated that cells of a strain of that bacterium were active in decarboxylating aspartic acid to yield β-alanine.

The importance of aspartic acid as a regulator of nitrogen assimilation from NO_3^- is further emphasized by its inhibiting effect on the reductase activity. A pronounced inhibition by NH_4^+ may also be due to this amino acid, since the cells in question have a significant aspartase activity and abundant fumarate is supplied by the citrate added to the test system.

Since the experiments point to aspartic acid and alanine as the key intermediates in the nitrogen assimilation from NO_3^- into amino acids we would expect to find a high aspartase activity and a low L-glutamic dehydrogenase activity in nitrate-grown cells. Such an adaption is also in fact followed by a complete repression of the L-glutamic dehydrogenase activity. In contrast to this, the aspartase activity is still present. The results exclude the L-glutamic dehydrogenase as the enzyme responsible for the nitrogen incorporation. The responsible enzyme could be the aspartase. However, the lack of repression by NH_4^+ and the facts that the aspartase activity is very low compared to that found in *E. coli*, and not increased as the result of an adaption to growth on NO_3^- seem to argue against the hypothesis. The lack of L-alanine dehydrogenase activity seems to be a general experience from Gram-

not seem to be influenced by the nitrogen source It is remarkable that the activity in *B anitratum* per mg protein is very much lower than that found in *E coli*, and not increased as the result of an adaption to growth on NO_3^-

TABLE 5

Measurements of Activities Corresponding to L glutamic Dehydrogenase L-alanine Dehydrogenase and Aspartase

Strain	Source of extract	L glutamic dehydro genase A_{340} mμ \ 10^3	L alanine dehydro-genase L alanine μg	Aspartase NH_3 mμg
B anitratum	Blood agar	27	0	22
B anitratum	NH_4^+ as N	31	0	23
B anitratum	NO_3 as N	0	0	26
B anitratum	glu as N	0	0	–
B anitratum	asp as N	–	0	25
N meningitidis	Blood agar	36	0	–
B subtilis	Blood agar	–	3 5	–
E coli K12	Blood agar	–	0	510

All activities are calculated per minute per mg of protein

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negative microbes except for the vibrios and possibly the pseudomonas group (24).

The activities reported in this communication refer to intact cells. The effect of the repressors and inhibitors tested could accordingly be on the penetration of the substrate. *Farkas-Himsley et al.* (2) suggested that the kinetics of the nitrate reduction by non-proliferating suspensions of *E. coli* could be ascribed to a permeability-barrier in old cells towards nitrate. The authors found a difference between intact cells and cell-free extracts which was suggestible of a substrate inducible permease.

It is clear that the results presented may adequately explain previous negative attempts to find a nitrate reduction in *B. anitratum* (19, 22, 10, 3). Previous discussions concerning the taxonomy of *B. anitratum* (B5W) have been concerned with the genus name. The present results indicate that future taxonomists should also pay attention to the epithet. The bacterium B5W is obviously not "anitratum" but rather "cryptonitratum".

SUMMARY

A reduction of nitrate to nitrite, and of nitrite to some unidentified product have been measured in suspensions of *Bacterium anitratum*.

The biosynthesis of the nitrate reducing system was repressed by several amino acids. Effective repressors were aspartic acid and alanine and amino acids biosynthetically related to these amino acids. Glutamic acid and amino acids directly synthesized from this amino acid did not act as repressors. The system was not repressed by the ammonium ion.

The activity of the nitrate reducing system was effectively inhibited by the ammonium ion. It was also inhibited by some amino acids. Potent inhibitors were aspartic acid, aspargine and cysteine. No inhibition was obtained with glutamic acid.

Extracts from *B. anitratum* grown with nitrate as nitrogen source have no activity corresponding to L-glutamic dehydrogenase. The same extracts possess aspartase activity. Extracts from cells grown with the ammonium ion as nitrogen source have activities corresponding to both enzymes. Extracts from *B. anitratum* have no L-alanine dehydrogenase activity.

It has been suggested that aspartic acid and alanine are the first amino acids in which the nitrogen from nitrate and nitrite is incorporated in *B. anitratum*.

The regulation of the nitrate reducing system by repression explains why previous attempts to demonstrate a nitrate reduction in *B. anitratum* have been negative.

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Acta path et microbiol scandinav 64 398 399, 1965

TRANSACTIONS OF THE FINNISH SOCIETY OF PATHOLOGY

Meeting February 5, 1965

A E Kortelangas BLOOD GROUP ANTIBODY ANTI–O

Several authors have recently published results of blood group antibodies with the specificity anti O or anti H The main difference between these two antibodies concerns the inhibition of the reagents with secretor saliva Anti H is clearly inhibited whereas anti–O is not Blood group antibody anti I shares some of the characters of 'anti–O' *Gold* has described in his recent paper 22 sera the reaction patterns of which varied between anti O and anti I He found most of his sera to react with compound antigens OI IO, Oi or iO, the relative amounts of the antigens determining seven different types of antibody

Among three own anti O reagents one called Kall was described This antibody was found during routine antenatal testing in an A_1B woman The A B and I antigens of her cells were normal and her cells reacted very weakly with anti H The antibody did not affect the cells of her baby This antibody was not able to detect O gene product from cells of the phenotypes A and B The reactions of the Kall antibody in different tests were described None of several secretor salivas tested inhibited the agglutination Surprisingly l fucose solution inhibited the antibody up to concentrations of 5 to 1 millimoles per litre None of other nine anti–O reagents had the same character whereas most anti H reagents were inhibited by l fucose A good although not definite correlation existed between the reactions with Kall antibody and lectin anti H (Ulex europaeus) Kall serum agglutinated to varying strength most of cord cells of group O investigated but some cord cells failed to react in spite of the fact that they all had almost identical reactions with anti I The speaker recommended Kall antibody to be called anti H irrespective the failing inhibition by secretor saliva

Kari Maatta PNEUMOCYSTIS CARINII PNEUMONIA

Pneumocystis carinii (p c) pneumonia is characterized by interstitial plasma cell infiltration of the lungs but in children with hypo or agammaglobulinaemia the alveolar septa are only slightly thickened and without plasma cell infiltration

The diagnosis of p c pneumonia cannot be made without the demonstration of p c in the pulmonary lesion Although all cases of interstitial plasma cell pneumonia in Finland have had clinical manifestations and been microscopically typical special histologic stains for demonstration of p c have never been made

In Turku in 1945–64 1586 autopsies have been performed on patients dead at the Children s Hospital of Turku University among these 29 cases of p c pneumonia In all of these cases using Gomori s silver methenamine stain numerous protozoa were found in the alveoles The first case occurres 1947 The cases in 1948–51 occurred in conjunction with institutional epidemics After 1951 sporadic cases have been diagnosed in 55 –56 63 and 64 Excepting the two last ones all cases have affected prematures or debilitated infants of ages 1 4 months The case in 1963 was

of a 2 year old girl who in addition to p c pneumonia, had generalized cytomegalic inclusion disease in 1964 (f a 7 month old boy, whose pulmonary changes corresponded histologically those which have been described in p c pneumonia cases connected with a or hypogammaglobulinaemia In the last case all virus as well as bacterial and fungus cultures were negative

Rauno Vantyjarvi A STUDY OF THE VIRAL ETIOLOGY OF RESPIRATORY INFECTIONS IN MILITARY TRAINEES PRELIMINARY RESULTS OF THE ROLE OF ADENOVIRUSES

The viral etiology of respiratory diseases in about *500 trainees in three training* centers during a period of 11 months was studied The preliminary results concerning adenoviruses in one of the study groups of 210 trainees were reported During the basic training period of about ten weeks from February 15th to April 26th 1964 a respiratory illness was reported in 37 per cent of the recruits 56 per cent of the reported cases were caused by adenoviruses according to the virus isolation and/or serological finding A significant rise in adenovirus antibody titre was noticed *during the basic training* period in 28 per cent of the recruits without reported respiratory illness The results indicate that 35 per cent of all recruits had adenovirus infection during the basic training period and 43 per cent of these infections were reported

After the basic training period only few respiratory infections were *reported and* they were not caused by adenoviruses

Meeting March 19, 1965

Received 19 iv 65

H Teir SOME ASPECTS OF THE PHYSIOLOGICAL FUNCTIONS OF NEUTROPHILS

Neutrophilic granulocytes have generally been of interest in connection with inflammations There is no definite idea of uniform line of thinking concerning their physiologic functions in the organism

Valuable *additions* have been made in the last few years to our information on the kinetics of neutrophils We know that an adult man produces daily about 150 $\times 10^9$ *cells* and that the life cycle of the cells is c 12 14 days The cells spend not quite a half of this time in the bone marrow They circulate for c 10–12 hours in the *blood* Half of the cells in the blood are marginated cells and do not circulate at all

There must consequently be a considerable number of extramedullary granulocytes in the various organs However we know that the organs *and tissues* have *relatively* few granulocytes especially neutrophilic granulocytes Our laboratory has been working on the subject of granulocyte elimination for the organism must after all eliminate daily as many granulocytes as it produces

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We have come to the conclusion that the gastrointestinal canal is the most important site of granulocyte elimination. Granulocytes, especially neutrophils, change very rapidly in the lamina propria of the intestine and are therefore difficult to identify. An average of 2–3 granulocytes are seen in epithelial diapedesis in the villi of 5 μ section of the human small intestine. Since the superficial area of the intestine is c. 19 sq. m, this gives some idea of the enormous total quantity involved. It has been proved by special methods that amounts equivalent to the production are really eliminated in the intestine.

These observations have automatically led to consideration of the physiologic functions of neutrophils. It is assumed that neutrophils perform the same functions in the normal organism as in an inflammation: they are phagocytosing and affect the tissues and organs via their enzymes. This probably happens principally when they are marginated cells. An extremely great number of cells is physiologically destructed in the organism: in an adult an estimated 600–700 g in 24 hours. Their degradation products must be eliminated. It is assumed that neutrophils also take care of these cleaning jobs in addition to lymphotic suction drainage and RES system. This is an immense task which calls for large numbers of granulocytes and on which the cells expend their energy fairly rapidly. In addition to the depletion of enzyme stores, neutrophils obtain through phagocytosis waste products that are dangerous for the kidneys. This load is carried by granulocytes into the intestine for elimination.

In addition to the cleansing function, granulocytes introduce into the tissues the most diversified enzymes and other biologically active agents in the same way as has been proved for eosinophilic granulocytes.

M. Kormano and *M. Niemi*: CADMIUM INDUCED TESTICULAR NECROSIS

The mechanism of the destructive action of cadmium in the testis was investigated using angiographic techniques. The results indicated that the primary locus of action is in the level of the intratesticular capillaries. Detailed description of the observations is published elsewhere in this journal (Acta path. et microbiol. scandinav. *63*, 513–521, 1965).

In order to find out whether an increase in the intratesticular pressure is responsible for the final events leading to a complete necrosis of the testis, a group of rats was subjected to incision of the tunica albuginea of one testis at the time of cadmium injection. This treatment was not able, however, to prevent *the necrosis*, which may be taken as an evidence against the proposed role of increased pressure.

Markku Seppälä: OCCURRENCE OF Tf B_{O-1} AND D_{Chi} IN FINLAND

The composition of the Finnish transferrin genes appears to be different from other white peoples studied. Preliminary results of 2923 random Finnish blood samples examined are as follows:

Tf CC 2779
Tf BC 66
Tf CD 78

Most of the BC types are identical with $B_{O-1}C$ and CD types with CD_{Chi}, which variants were found by Dr. *A. G. Bearn*, to whom Finnish BC and CD samples were sent. 8 of the specimens are B_2C. Family studies have revealed a homozygote which is indistinguishable from B_{O-1}.

Acta path et microbiol scandinav 64 401–406, 1965.

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(Head. Reidar Eker, M D)

PLEXIFORM LESIONS OF PULMONARY ARTERIES

By

HARALD AARS

Received 3 xii 64

The plexiform lesion[1] is a peculiar and disputed pathological phenomenon mainly found in cases of severe and long standing pulmonary hypertension, consisting of nests of small vessels inside dilated segments of muscular pulmonary arteries (for general references, see *Wagenvoort, Heath & Edwards* 1964.

The pathogenesis of the lesion is still uncertain. Most authors consider it a thrombotic response to the raised pulmonary artery pressure, followed by recanalization. The anatomical features are more clearly defined, although not finally settled. Functionally, the lesions will increase the vascular resistance of the pulmonary circulation.

The structure of the plexiform lesions has been investigated in serial sections and reconstructions by *Wagenvoort* 1959, *Naeye & Vennart* 1960 and *Moschcowitz, Rubin & Strauss* 1961, the latter giving a 3-dimensional illustration of the lesion. In the present paper a slightly different technique, also utilizing serial sections and reproductions, has been applied, in order to clearly demonstrate the anatomical structure of the lesion and to see whether this in any way could elucidate the pathogenic mechanisms.

CASE HISTORY

A 34 year old woman had for nine years shown signs of pulmonary hypertension, starting with haemoptysis and ending in severe right heart strain and failure the last two years (12).

Except for recurrent episodes of syncope when she was 10 to 16 years old, she had previously been healthy. Following a right heart catheterization and angiocardiography in 1950 a diagnosis of primary pulmonary hypertension was made. She was treated with anticoagulants, later also with diuretics. The catheterization was repeated in 1960, the pulmonary artery pressure then being 132/60 mm Hg as opposed to her systemic pressure of 110/75 mm Hg. She died in August 1961 in right heart failure.

A partial autopsy in another hospital revealed an incomplete rupture of the pulmonary artery two to three cm above the valves, haemorrhagic changes in the surrounding adventitia and atherosclerosis of the main pulmonary arteries and aorta. The heart weighed 610 g, the right side being particularly enlarged. Sections were taken from the lungs, heart, liver, pulmonary artery and aorta.

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[1] Also called glomoid or glomerulus like structures or lesions.

I wish to thank Mrs *F. Horn* for the reproductions and Miss *I. Gjoen*, University of Oslo, for the drawings.

MICROSCOPIC EXAMINATION

The pulmonary artery trunk and main branches showed atherosclerotic patches, and a hypertrophic medial layer with aorta-like configuration of the elastic tissue The partial rupture of the pulmonary artery traversed the apparently normal media, being covered only by the thickened adventitia

The small and medium-sized pulmonary arteries were found to have concentric or cushion-like intimal thickenings The media was mostly hypertrophic. A few small arteries were occluded, with a thin and fragmented media, lymphocytic infiltration and recanalization

Several plexiform lesions were found, consisting of nests of small vessels inside medium-sized muscular pulmonary arteries shortly after branchings A tortuous afferent artery ended in a dilated and thin-walled body of external diameter about 200 μ, inside which numerous endothelial-lined vascular channels were found These were mostly extremely narrow (diameter down to 5 μ), and were separated by muscular fibres and connective tissue The efferent vessels were thin-walled, dilated arterioles and pre-capillaries Some of the plexiform lesions were fibrotic or hyalinized

No signs of emboli or recent thrombi were found The other pulmonary vessels and the pulmonary parenchyma appeared normal The exact location of the small pieces of lung tissue is unknown

The wall of the right ventricle was hypertrophic, the liver showed early cardiac cirrhosis

RECONSTRUCTION

One of the plexiform lesions was serially sectioned at 7 μ The sections were enlargeed by projection (165 times enlargement) and drawn Corresponding pieces of celluloid 0 5 mm thick were cut to shape and put together in two models One model shows the outside of the parent artery, the branch with the dilated body and the small vessels eventually leaving it, the other shows the lumens of the artery and the plexiform lesion The models, one of which is shown in Fig 1, provide 3-dimensional illustrations of the plexiform lesions Because of their transparency and laminar construction, they are unsuited for reproduction Therefore, an artist's drawings of the model are presented (Fig 2). Fig 3 is a microphoto of a section through the middle of the lesion

The vascular channels forming the nest were found to communicate with each other more or less like threads in a net, most of them eventually ending in thin-walled, narrow or dilated vessels piercing the arterial wall surrounding the nest, but some of them ending in a cul-de-sac Although the efferent vessels were rather similar to veins, they were in fact shown to be arterial, ending in ordinary capillaries and unconnected to the bronchial circulation



Fig 1
A reconstructed model of a plexiform lesion × 165 This model is the original for the drawing in Fig 2 B



Fig 2
Artist s drawings of the reconstructed models
A The outside of the parent artery and plexiform body
B The lumens of the artery and vessels constituting the plexiform lesion

DISCUSSION

The pathogenesis of the plexiform lesions has been debated for several years They might be congenital (9) or acquired, secondary to pulmonary hypertension Although rare below the age of two years, the lesions have been seen even in infants merely eight weeks old (14), and to a certain extent the first theory conforms with this and with the

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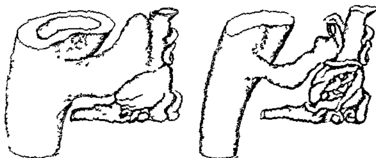
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A *B*

Fig 2

B

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Fig 3
Microphoto through the middle of the lesion Haematoxylin eosin, × 118

lack of plexiform lesions in cases of mitral stenosis with pulmonary hypertension. This point, however, is explained by *Wagenvoort* (13) In this condition, the elastic pulmonary arteries are contracted, thereby protecting the muscular arteries from some of the effects of hypertension In hyperkinetic pulmonary hypertension, the elastic arteries are dilated, the spasm occurring in the muscular arteries. Some authors (11) have pointed out the similarity between segmental occlusive vascular anomalies in the lungs of newborn and the plexiform lesions in older subjects. These anomalies consist of intimal cushions or proliferations and a defective media, and might develop into plexiform lesions Usually, however, the age distribution of the plexiform lesions and the positive correlation to hyperkinetic pulmonary hypertension (5, 14) are considered to favour an acquired origin of the lesion. This is further supported by the development of plexiform lesions following anastomosis of the systemic and pulmonary circulation (4, 6, 15).

A thrombotic mechanism is considered by several authors *Wagenvoort* (13) suggested that the arterial spasm might lead to necrosis, which again might cause thrombosis, and that the plexiform lesions were recanalized thrombi Others, however, have found the lungs remarkably free from thrombi, and the lesions lacking the morphological characteristics of canalized thrombi (9) One difficulty has been to explain why the lesions are always located just distal to arterial branchings. *Downing* and associates (4) pointed out that this location coincided with an area of turbulence, and suggested that this turbulence might cause thrombosis.

Embolic mechanism are often discussed, but usually denied because

of the uniformity in size of the affected arteries and the lack of known origin of the emboli. Experimentally, plexiform lesions have been produced in rabbits by recurrent air emboli by *Wright* (17). It is interesting to note that one of the patients of *Naeye & Vennart* (10) was a 31 year old man who developed primary pulmonary hypertension nine years after several episodes of decompression sickness.

The possibility of the plexiform lesions being arterio-venous anastomoses or pulmonary-bronchial artery shunts, has generally been ruled out by investigations of serial sections (1, 2, 7, 13), although occasional examples have been found (3, 8). The present reconstructions failed to show such connections.

The technique employed in the present investigation is well suited to demonstrate the structure of the plexiform lesion. As the procedure is rather time consuming and elaborate, it was not thought worth while to apply it to the number of lesions required to reach a final conclusion. Instead the models are presented as examples illustrating the technical possibilities and the aspect of the one of these lesions.

The lesion is composed of a nest of actual vessels, and even if a few of them come to a dead end, it is very difficult to visualize their lumens being formed by irregular endothelial proliferations, a possibility mentioned by *Edwards* (5). Other authors have also found a few blind processes, but most of the channels have been traced to capillaries (10, 13).

Although reconstructions of canalized thrombi are unknown to the author, it is felt that the plexiform lesion in this case probably represents a late stage of such mechanism, and the simultaneous occurrence of organized thrombi with inflammation and more typical canalization lends further support to this. The finding of a foetal type of pulmonary artery elastic tissue points to the existence of pulmonary hypertension from birth (16), but does not necessarily warrant a congenital origin of the plexiform lesions.

SUMMARY

The occurrence of plexiform lesions in the pulmonary arteries is discussed with special emphasis on their pathogenesis and structure. Such lesions were found in a 34 year old woman who died from primary pulmonary hypertension. Through serial sections and reconstructions in the form of actual models, a clear illustration of the lesion is given.

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The University Institute of Pathological Anatomy, Copenhagen, Denmark

CLASSIFICATION OF ENDODERMAL SINUS TUMOUR (MESOBLASTOMA VITELLINUM) AND SO-CALLED "EMBRYONAL CARCINOMA" OF THE OVARY

By

Gunnar Teilum

Received 3 ii 65

Comparative studies of tumours of the ovary and testis (35-38) showed that neoplasms of germ cell origin (gonocytoma, germ cell tumours) are in great part homologous in the male and female While the occurrence of ovarian dysgerminoma, teratoma as well as very rare cases of teratomatous choriocarcinoma all were well known, the development in the ovary of so-called "embryonal carcinoma" was not appreciated (cf *Friedman* (11), *Sternberg* (33)) A specific ovarian tumour of germ cell origin showing a close histologic resemblance to certain types of testicular "embryonal carcinoma" was first recognized and distinguished from tumours of entirely different origin by the comparative studies of homologous tumours (*Teilum* (35, 36, 38))

In the ovary this tumour had a distinctive microscopic pattern with stellate mesothelial cells forming a loose network with wide meshes or a system of communicating cavities or channels Another characteristic feature was the occurrence of perivascular formations with mantles or star-like halos of cells of epithelial appearance and surrounded by capsular sinusoid spaces While this type of ovarian tumour previously was misinterpreted as "mesonephroma" and at the same time confused with different tumour entities (*Schiller* (28, 29)) or misdiagnosed as e g "endothelioma of ovarian anlage" (17), angiosarcoma, angioreticuloma or unspecific carcinoma, it was undoubtedly classified as an ovarian counterpart of "embryonal carcinoma" in the testis, and like this it was thought to arise from totipotent germ cells The germinal origin conformed with the occasional presence of areas showing dysgerminomatous, teratoid or trophoblastic structures (35)

In subsequent studies the author presented a more specific interpretation of the essential elements in this tumour type in the ovary, which were found to reproduce characteristic stages in the morphogenesis of extra-embryonic membrane structures of the early embryo (36) and

yolk sac endoderm lining sinusoid spaces (38) and mimicking the "endodermal sinuses" (Duval) of the rat placenta

This extra-embryonic membrane tumour was therefore designated an *extraembryonic mesoblastoma* (36), or an *endodermal sinus tumour* (38) of germ cell origin These concepts on histogenesis have gradually been accepted in their common or more specific form, *e g* by *Santesson & Marrubini* (26), who reported the first large series of 17 cases, *Cabanne* (3, 4), *Martin et al.* (18), *de Brux et al* (2), *Saphir* (27), *Masson* (19), *Scully* (30), *Sternberg* (33) and *Neubecker & Breen* (20), who recently studied 27 cases and again stressed the analogy to the extra-embryonic mesoblast of the early embryo It is of interest that also germinal tumours in young children (*Huntington et al* (16) in many instances exhibit the pattern characteristic of the endodermal sinus tumour in the ovary, while the testicular "embryonal carcinomas", that occur in adults, are usually of a more variegated type and more frequently associated with teratoid or trophoblastic differentiation

In this paper evidence is presented that so-called "embryonal carcinomas" of the ovary are principally represented by this specific type of germ cell tumour showing a selective overgrowth of yolk sac endoderm intimately related to the exocoelomic mesoblast. Comparative studies show that various histologic patterns in the tumour reproduce stages in the development of the extra-embryonic mesoblast-vitelline structures of early embryos

The significance of the unilateral growth of mesoblast-yolk sac derivatives in endodermal sinus tumours of the human ovary is discussed in the light of recent experimental studies on conversion of transplantable testicular and ovarian teratomas of mouse to the ascites form (*Pierce & Dixon* (22 24))

HISTOLOGICAL APPEARANCE OF EXO COELOMIC MESOBLAST-YOLK SAC STRUCTURES IN THE OVARIAN TUMOUR

The general histology of the tumour is characterized by the following patterns corresponding to the various degree of differentiation.

1 Areas of stellate mesodermal cells forming a *loose vacuolated net-*

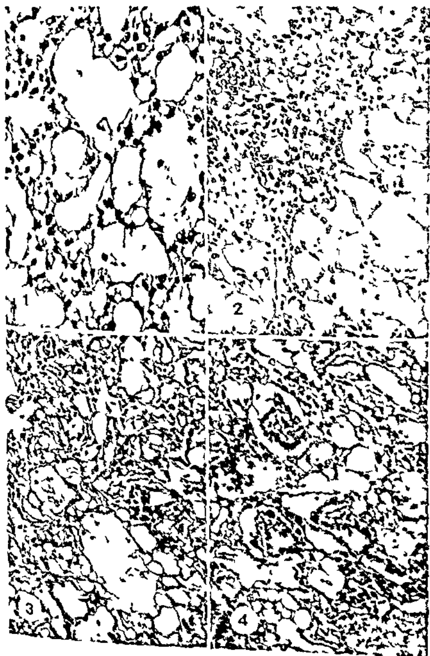
Figs 1–4

Fig 1 Ovarian tumour (case 1) Loose vacuolated network with wide meshes or cystic spaces (H & E × 260)

Fig 2 Ovarian tumour (case 2) Aggregates of undifferentiated embryonal cells and cystic spaces lined with flat mesothelioid cells (H & E × 140)

Fig 3 Ovarian tumour (case 3) Yolk sac derivatives forming a vacuolated and small cystic pattern (H & E × 110)

Fig 4 Tumour of infantile testis (case 7) exhibiting the same small cystic, vitelline pattern as the ovarian tumour as well as endodermal sinus structures (H & E × 110)


1
2
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work with wide meshes or cystic spaces (Figs 1–4) sometimes lined by flat mesothelial cells and showing foci of active hemopoiesis in the underlying capillary spaces. The meshes often contain hyaline, acidophilic and PAS-positive globules or mucoid precipitates. The more compact areas may show star like halos or mantles of cells surrounding the larger capillaries. The characteristic loose pattern resembling the "*magma reticulare* or *the extra embryonic mesoderm* of the exocoelom (Figs 5 and 6) led first to the recognition of the *mesoblastic* nature of this ovarian germ cell tumour, i e mesoblastoma extra embryonale (36)

Cases of ovarian mesoblastomas were later studied by *Cabanne* (3 4) *Martin et al* (18) *de Brux et al* (2) and *Masson (19)*

2 *Endodermal sinus pattern* In more differentiated areas are seen groups of peculiar perivascular formations (Figs 7 and 8) consisting of *a mesodermal core with a capillary in its centre and covered with a* visceral layer of cylindric cells of epithelial appearance. The surrounding capsular sinusoid space is lined by a single (parietal) layer of flat cells with prominent nuclei. The general patterns often resemble a complicated labyrinth (Fig 11) of communicating cavities and channels with papillary processes. *Teilum* (38) interpreted these perivascular sinusoid structures—regarding the general architecture appearance in cross and longitudinal sections and the type of lining cells—is equivalent to the endodermal sinuses (Fig 9) i e, embryologically well defined structures which are prominent in the rat placenta (*Duval* (7)) They are diverticula of yolk sac endoderm that expands and dissects around the vessels of the extraembryonic vascular mesenchyme and are formed half of a layer of visceral (proximal) endoderm and half of parietal (distal) endoderm and Reichert's membrane (Fig 17) In these specific structures as in the human tumours the yolk sac endoderm is intimately connected with the exo-coelomic mesoblast. The embryological relationship between primitive mesoderm and endoderm during stages in development of the yolk sac will be considered later

3 Compact aggregates of small *undifferentiated neoplastic embryonal cells* which are considered analogous to the masses *of undifferentiated* cells visible during the early stage of development of the embryo (Fig 12) Certain sections of the tumour show such areas merging with the *reticular network of extraembryonic mesoblast* (Fig 2) or the endodermal sinus pattern (Figs 20 21)

Figs 5–6

Fig 5 Ovarian tumour (case 5 Areas showing characteristic yolk sac (endoblast) xtra

Fig 6 el al

er st c

festoon shaped structures and individual cysts lined with in part mucinous epithelium (H & E × 200

5

6

work with wide meshes or cystic spaces (Figs 1–4) sometimes lined by flat mesothelial cells and showing foci of active hemopoiesis in the underlying capillary spaces The meshes often contain hyaline, acidophilic and PAS-positive globules or mucoid precipitates The more compact areas may show star like halos or mantles of cells surrounding the larger capillaries The characteristic loose pattern resembling the "magma reticulare" or the extra embryonic mesoderm of the exocoelom (Figs 5 and 6) led first to *the recognition of the mesoblastic* nature of this ovarian germ cell tumour, i e, "mesoblastoma extra embryonale" (36)

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Figs 5–6

festoon shaped structures and individual cysts lined with in part mucinous epithelium (H & L × 290)



Figs 7 9

Fig 7 Ovarian tumour (case 4) Areas showing the characteristic endodermal s nus pattern in cross and longitudinal ections (V Gieson s stain × 210)

Fig 8 Ovarian tumour (case 4) showing groups of endodermal sinuses represent ng yolk sac endoderm that expands and dissects around the vessels of the vascular extra embryonic mesoderm (V G × 190)

Fig 9 Endodermal sinus of rat placenta i e yolk sac liverticula formed half of a layer of visceral endoderm and half of par etal endoderm and Reichert s membrane (H & E × 190)

4 *Cystic structures* may form a regular arrangement of small cavities lined by a layer of flat cells with protruding nuclei continuous with the parietal lining of the endodermal sinuses, or individual cysts showing a clear columnar in part mucinous epithelium may occur in the loose myxoid stroma with mesoblastic mesothelial like elements and early differentiation of yolk sac endoderm forming characteristic festoon shaped configurations (Figs 6 and 11) I consider these endodermal cysts (Fig 5) equivalent to the yolk sac (endoblast) vesicle of the early embryo (Fig 12) and this part of the tumour (Figs 5 and 6) demonstrates clearly the intimate relation between the extra embryonic mesoblast and yolk sac structures to be discussed under comparative morphogenesis

Occasionally the predominant part of these vitelline tumours shows a myriad of yolk sac (endoblast) vesicles (Fig 13) lined with a columnar or cuboidal epithelium showing transition to a layer of flat mesothelial like cells The individual cavities are varying in form and size and show invaginated processes and protrusions into the lumen Often the distal parts of the vesicles covered with mesothelial cells are constricted off from the portion covered with in part mucinous epithelium—thus making a smaller vesicle— reflecting the embryologic conversion of the primary yolk sac into the secondary sac (Compare Figs 14 A and B with Fig 16 B) Cystic spaces clothed with mesothelioid cells are considered comparable with exocoelomic vesicles representing remnants of the primary yolk sac (Compare Fig 15 with Fig 16 C)

COMPARATIVE MORPHOGENESIS AND INTERPRETATION

In my previous communications (36 38) the specific elements in this ovarian germ cell tumour were interpreted as extra embryonic mesoblast connected with derivatives of yolk sac endoderm which reproduced the structure of the well defined endodermal sinuses In accordance with this additional points of resemblance to stages in the embryological development of the primary and secondary yolk sac and the covering extra embryonic mesoderm should be considered

In human development (13 32) the yolk sac shows certain peculiarities apparently due to the precocious development of the extra embryonic mesoderm This mesoderm seems to be derived in part at least from the inner surface of the trophoblast and differentiates before the

... a layer of flattened cells mesothelial in character representing the inner limiting layer (Heuser's exocoelomic membrane) of the extra embryonic mesoderm which is in continuity with the primitive endoderm around its margins and enclose the primary yolk sac (Fig 16)

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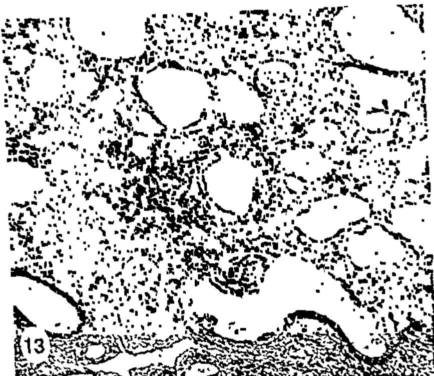


Fig 13

Ovarian tumour (case 5) Section showing numerous yolk sac (endoblast) vesicles lined with cells of columnar or mesothelioid type with transitions from one to another (H & E × 90)

Whether this "mesoderm" is truly mesodermal is still in dispute, as it may be formed by an extension from the disc endoderm by mesothelial-like cells On the ca 11th–12th day of development the extra-embryonic mesoderm has increased in amount and forms a loose reticulum, the magma reticulare (Fig 16 A) Later the small cavities in this reticulum enlarge and become confluent to form the extra embryonic coelom except in an area where the amnion remains attached to the trophoblast The part of the mesoderm covering the yolk sac is called the extra-

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Figs 10–12

Fig 10 Ovarian tumour (case 6) Endodermal sinus showing intracellular PAS positive diastase resistant hyaline globules in the lining yolk sac cells (H & E × 240)

Fig 11 Ovarian tumour (case 5) showing complicated labyrinth of communicating [illegible] nd numerous endodermal [illegible]

Fig 12 [illegible] (*below*) in contact with [illegible] s of undifferentiated cells [illegible] × 300)

14a

14b



Fig 15

Ovarian tumour (case 5) showing the same process of conversion as Fig 14 The multiple small cavities lined with a thin layer of mesothelioid cells are considered homologous of exocoelomic vesicles some of which may represent vestigial remnants of the primary yolk sac (cf Fig 16 C) (H & E × 320)

embryonic splanchnopleuric (visceral) mesoderm (Fig 16 C) The primary yolk sac soon decreases in relative size owing to proliferation of the mesoderm and the development of the extra embryonic coelomic spaces and become converted into the secondary yolk sac This is apparently brought about by portions pinching off from the primary sac (Fig 16 B), thus making a smaller vesicle Mesothelioid cells adjacent to the disc may be transformed into cuboidal cells and this portion of the primary sac constricted off from the distal portion to form the secondary sac (Fig 16 C) Remnants of the distal portion of the primary yolk sac persist as exocoelomic vesicles lined by mesothelial like cells

Fig 14

(a and b) Ovarian tumour (case 5) Blastocyst like yolk sac ves cles showing invaginated protrusions into the lumen and constriction of the wall of the cysts with portions pinching off from the primary cavitity and thus reflecting the embryologic conversion of the primary yolk sac into the secondary sac (cf Figs 15 and 16 B) (H & E × 210)

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Altogether it will appear that ovarian tumours belonging to the

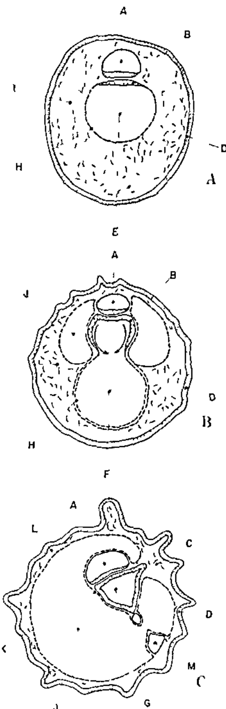


Fig. 16

(A–C). Diagrams illustrating stages in development of the human yolk sac and relation to extra embryonic mesoblast (Adapted from 13) and 32) The letters indicate: A, amniotic cavity, B, embryonic endoderm; C, connecting stalk, D, cytotrophoblast; E, primary yolk sac; F conversion of the large primary yolk sac of earlier stages into the smaller secondary yolk sac, G, secondary (definitive) yolk sac; H, Heuser's exocoelomic membrane; I, loose reticulum of extra-embryonic mesoblast (magma reticulare), J, extra-embryonic coelom; K, extra embryonic splanchnopleuric (visceral) mesoderm; L extra-embryonic somatopleuric (parietal) mesoderm, M, vestigial remnants of the primary yolk sac forming vesicles in the exocoelom

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B, shows the process by which the large primary yolk sac of earlier stages is converted into the smaller secondary sac (for comparison with the findings in the human ovarian tumour (Figs 14 15) suggesting a "pinching off" of the primitive abembryonic portion

C to show the secondary (definitive) yolk sac and vestigial remnants of the primary sac forming vesicles in the exocoelom (cf ovarian tumour Fig. 15) The extra-embryonic mesoderm is now separated into two layers, a parietal lining the trophoblast and the sides of the amnion and a visceral layer covering the yolk sac and called the extra-embryonic splanchnopleuric mesoderm Evidence has been presented to show that evaginations and funnel like formations of hemopoietic splanchnopleuric yolk sac homologous with the definitive yolk sac are reproducing the endodermal sinuses *i e* diverticula of yolk sac endoderm that "dissects" around the larger vessels of the extra-embryonic mesoderm (Cf Figs 7 10)

In the light of the embryological development it can not surprise that in the more undifferentiated parts of the tumour it is often impossible to make a clear distinction between mesodermal and endodermal derivatives The intimate relationship between exocoelomic mesoblast resembling the magma reticulare and cystic and festoon-shaped vitelline structures in the tumours is shown in Fig 6 Altogether it is possible to correlate the various histological patterns in the tumours with stages in the early embryological development of these foetal membranes Thus

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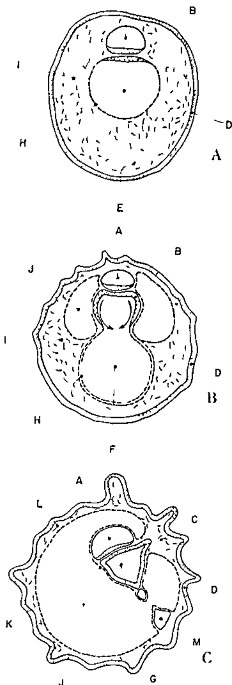


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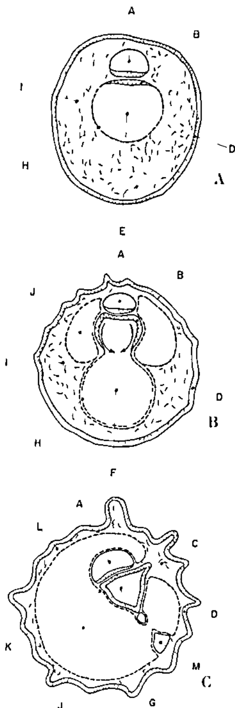


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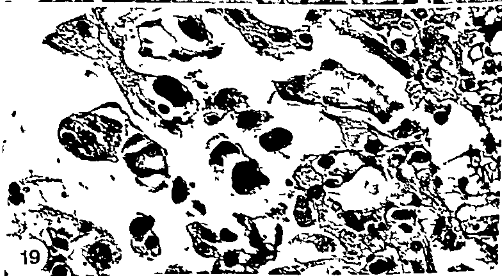
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general class of so called "embryonal carcinomas" show relative little variation from tumour to tumour and the various histological patterns are clearly defined as a *selective overgrowth of exo coelomic mesoblast and vitelline endoderm* The presented interpretation of these endodermal sinus tumours (mesoblastoma vitellinum) in the human ovary is supported by the recent experimental studies by *Pierce & Dixon* (22-24), who *during conversion of a transplantable testicular teratoma of* the mouse to the ascites form found a simplification of the tumour pattern from teratocarcinoma containing many elements to a yolk sac carcinoma intermingled with areas of *well formed visceral yolk sac* arranged in small cystic spaces and sometimes in papillary tufts Gradual transitions could be traced between the cells of the carcinoma with hyalin and this visceral yolk sac, indicating a *related origin of the* cells A few small cysts were formed by a layer of cells with dark, almost pyknotic nuclei and flattened cytoplasm arranged on the inner surface of a dense band of hyaline material The appearance was suggestive of parietal yolk sac endoderm resting on Reichert's membrane The teratocarcinoma employed was LS 402 VI, the tumour arose spontaneously in the testis of a strain 129 mouse and was developed as a transplantable line by *L C Stevens* of the Roscoe B Jackson Laboratory (34) *Also an ovarian teratocarcinoma in mice underwent ascitic con*version (22) Once the tumour had lost the ability to produce certain differentiated tissues as a result of the conversion, the tumour never *regained the ability to produce the tissue*

Fawcett et al (9 10) showed, as a result of growing fertilized mouse ova beneath the renal capsule, the presence of cells embedded in hyalin that looked remarkably similar to those described by *Pierce & Dixon* (23, 24), *and they considered them to be parietal yolk* sac in origin

The experimental work of *Pierce & Dixon* is of great interest for many reasons and may be directly applied to human teratology The experimentally evoked change from teratocarcinoma to a yolk sac carcinoma

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Figs 17 19

Figs 17 A portion of the yolk sac wall of the rat placenta Above the cleft like cavity are vascular villi of the splanchnopleure lined with proximal (visceral) endoderm consisting of a regular layer of cylindrical or cuboidal cells The lower limiting layer is the distal (parietal) yolk sac endoderm displaying a flat epithelium with projecting nuclei resting on Reichert's membrane Vascular spaces of the placental labyrinth are visible in the lower right corner (H & E × 420)

Fig 18 Ovarian tumour (case 1) showing the neoplastic yolk sac epithelium resting on dense bands of hyalin as well as tumour cells embedded in hyalin (H & E × 420)

Fig 19 Ovarian tumour (case 1) Areas showing a *close relation* between tumour cells resting on bands of hyalin and focal accumulation of PAS positive hyaline globules suggesting a common origin from the endodermal tumour cells lining the spaces (H & E × 420)

reflects the unilateral development of mesoblast-vitelline structures in human ovarian tumours The significance of the differences in morphologic differentiation of ovarian and testicular "embryonal carcinomas" in human subjects is not clear Possibly a continued selection of those cells best able to survive—as suggested for the transplantable mouse tumours (24)—may be a factor of importance The experiments indicated that it is possible to select populations of embryonal carcinoma cells with uniform, but extremely limited, potential for differentiation (22) According to *Pierce* this would suggest that embryonal carcinoma cells in a teratocarcinoma are a heterogeneous collection, some multipotential, some determined, but all cancerous

The selective mesoblast-vitelline overgrowth may also occur in exceptional cases of primary testicular tumours in adults (Fig 21), but far more frequently intermingled with a variety of different histological patterns including immature or adult teratoid structures, ectoblastic and trophoblastic elements In such cases, however, a unilateral development of mesoblast-vitelline derivatives is sometimes found in the metastases

Secretion of hyaline material In several cases of vitelline tumours of the human ovary and testis I found dense bands of hyaline material in direct contact with the surface of yolk sac epithelium (Fig 18), and focally neoplastic cells resembling parietal yolk sac were found embedded in hyalin similar to *Pierce & Dixon*'s observations (24) during the conversion of the teratocarcinoma in mouse to the ascitic form These authors assumed that the hyalin is probably analogous to the Reichert's membrane (cf Fig 17) Although this structure only occur in rodentia and embryologists are divided in their opinion concerning the cell of origin of this membrane (39), the findings in these human tumours suggest the idea that the "carcinoma with hyalin" is of yolk sac origin (24, 25) An accumulation of PAS-positive hyaline globules (or "boules hyalines") is a characteristic finding in human ovarian and testicular tumours of the mesoblastoma-type (19) Such round or oval hyaline globules were present both intracellularly and extracellularly in all of the tumours in the series described by *Neubecker & Breen* (20), and I have observed them in all of my cases While the significance of these bodies has not been established or discussed, the present studies showed in several areas of the human tumours a close relation between tumour cells resting on a dense band of hyalin and a focal accumulation of the PAS-positive globules in large numbers (Fig 19) The findings suggested that these two types of hyalin had a common origin representing a product of secretion of the tumour cells lining the spaces Obviously, large spherical drops of a peculiar acidophil substance frequently accumulate in the body of the cells When the cytoplasm disintegrates these inclusions are set free and remain between the elements of the tumour in the form of "hyaline globules

The finding of the PAS positive, diastase-resistant hyaline bodies



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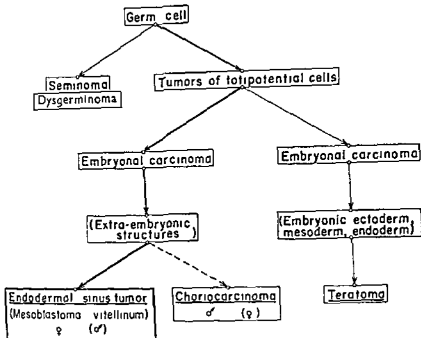


Fig 22

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histogenesis and interrelationship of ovarian and testicular tumours derived from germ cells as suggested in this comparative study is indicated in the diagram Fig 22

It should also be noted that several histologic patterns, of embryonal testicular tumours, described as "mesodermal type" and "unusual epithelial forms" of embryonal carcinoma (5, 6) actually represent extra-embryonic rather than embryonic forms, although in the adult there is much overlapping between the various forms of embryonal testicular tumours Neoplasms in the ovary described as "embryonic forms" (30) of embryonal carcinomas without evidence of teratoid differentiation are mostly undifferentiated extra-embryonic forms showing the characteristic pattern of mesoblastoma When it is maintained, that the mesoblastoma or endodermal sinus tumour probably is only a special form of embryonal carcinoma' (33) this is in agreement with the presented interpretation, but at the same time it should be realized that the selective development of these specific patterns represents the typical manifestation of an ovarian germ cell tumour belonging to this general class

Since *Peyron* s original discovery (21) several workers have observed embryoid bodies in embryonal testicular tumours Many of the embryoid

intracellularly in the cells of the endodermal sinuses (Fig 10) as well as in the "mesothelial" meshwork and the cells of epithelial appearance covering the individual yolk sac vesicles also suggested a close relationship between these cell types in accordance with the histogenetic interpretation

Pierce et al (25) also observed small intracytoplasmic droplets of PAS-positive, diastase-resistant material in the tumour of the mouse, which they identified electron microscopically and immunochemically as a yolk sac carcinoma

INTERRELATIONSHIP OF ENDODERMAL SINUS TUMOUR (MESOBLASTOMA VITELLINUM) AND SO CALLED 'EMBRYONAL CARCINOMAS OF TESTIS

In former times the terms testicular seminoma and embryonal carcinoma were often used interchangeably and often misapplied 'Embryonal carcinoma" in the testis has been considered as much a conceptual as a morphologic entity (5, 6) and have been defined as highly malignant tumours composed of multipotential, entirely undifferentiated, neoplastic embryonal cells However, also tumours showing early differentiation toward somatic (epithelial or mesodermal) or trophoblastic cell forms have been included, and tumours showing areas of glandular formations have sometimes been designated "embryonal adenocarcinomas" without making attempts to a more specific interpretation Usually the embryonal tumours in the adult testis show a multiplicity of morphological pattern with variations, which are not characterized histologically Although the designation "embryonal carcinoma" for practical use is applicable for these complex germ cell tumours in the testis, which comprise a fairly homogeneous *clinical* group, it is not a sufficient basis for the histologic characterization of the ovarian counterpart, which are usually large tumours exhibiting—as it will appear—a selective, unilateral overgrowth of extra-embryonic primitive mesoblast connected with derivatives of yolk sac endoderm lining sinusoid spaces or veritable endodermal vesicles or small cysts

Therefore it can not surprise that the germ cell origin of the ovarian tumour for long remained unrecognized, and that these cases were misdiagnosed and confused with tumour types of different origin, such as mesonephroma, endothelioma, mesothelioma, angiosarcoma etc While the embryonal carcinoma cell in the testicular tumours generally is considered capable of further differentiation into either mature tissue types reproducing adult tissues of teratoma (or teratocarcinoma) or into choriomatous elements (choriocarcinoma), the development of other types of *extra-embryonic* membrane structures, such as mesoblast and yolk sac endoderm, *i e* the dominating elements in the great bulk of the homologous counterpart in the ovary, and also present in many testicular tumours, has not at all received consideration The

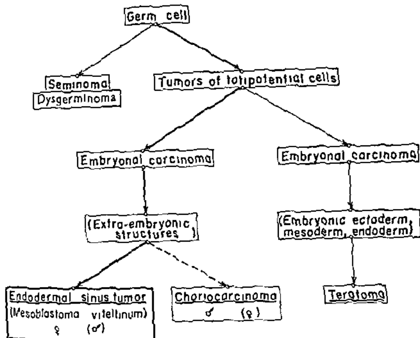


Fig 22

histogenesis and interrelationship of ovarian and testicular tumours derived from germ cells as suggested in this comparative study is indicated in the diagram Fig 22

It should also be noted that several histologic patterns, of embryonal testicular tumours, described as "mesodermal type" and "unusual epithelial forms" of embryonal carcinoma (5, 6) actually represent extraembryonic rather than embryonic forms, although in the adult there is much overlapping between the various forms of embryonal testicular tumours Neoplasms in the ovary described as "embryonic forms" (30) of embryonal carcinomas without evidence of teratoid differentiation are mostly undifferentiated extra embryonic forms showing the characteristic pattern of mesoblastoma When it is maintained, that the mesoblastoma or endodermal sinus tumour probably is only a special form of "embryonal carcinoma" (33) this is in agreement with the presented interpretation, but at the same time it should be realized that the selective development of these specific patterns represents the typical manifestation of an ovarian germ cell tumour belonging to this general class

Since *Peyron*'s original discovery (21) several workers have observed embryoid bodies in embryonal testicular tumours Many of the embryoid

bodies differ from normal embryos, but others are remarkable embryoid (8) In an excellent paper *Simard* (31) described for the first time the occurrence of unnumerable blastocysts in a malignant ovarian teratoma analogous to Peyron's observation While the occurrence of such embryoid bodies, composed of ectodermal and endodermal vesicles with mesodermal elements between them, is an exceptional finding in embryonal ovarian tumours, a selective development of the endodermal vesicles as described in this paper (Figs 13–15), actually represents a typical form of vitelline tumour, showing multiple cavities comparable with the vesicula vitellina and surrounded by the extra-embryonic mesoblast

The experimental studies of *Pierce & Dixon* (23–24) and *Stevens* (34) have shown that the teratomatous embryoid bodies of strain 129 mice develop from undifferentiated embryonal cells of the tumour and not directly from germ cells The fact that embryoid bodies are formed in teratomas indicates (Stevens) that teratomatous stem cells share a potentiality of a germ cell, and this supports the contention that teratomas are of germ cell origin, but it is therefore incorrect to conclude that teratomas develop as a result of a parthenogenetic process (34)

RELATION TO OTHER GERM CELL TUMOURS IN THE OVARY

In several of the reported cases the ovarian endodermal sinus tumour has been associated with areas of other germ cell tumours, such as dysgerminoma, adult teratomas or dermoid cysts In the case reported by *Bettinger & Jacobs* (1) the patient had a typical endodermal sinus tumour in the right ovary and a benign dermoid cyst in the left More rarely small areas suggestive of choriocarcinoma have been observed Of special interest is the association of endodermal sinus tumour with gonadoblastoma (dysgenetic gonadoma) showing typical foci of calcification (26)

CLINICAL DATA

Material from the following 8 cases of yolk sac tumour was used in this comparative embryological study

A *Cases (No 1 6) of vitelline tumours in the ovary*

Case 1 (Figs 1, 6 18 and 19)	pt aged 22 years
Case 2 (Fig 2)	pt aged 17 years
Case 3 (Fig 3)	pt aged 15 years
Case 4 (Figs 7 and 8)	pt aged 35 years
Case 5 (Figs 5, 11, 13, 14, 15 and 20)	pt aged 16 years
Case 6 (Fig 10)	pt aged 35 years

B *Vitelline tumour in the infantile testis*

Case 7 (Fig 4)	pt aged 12 months

C *Vitelline tumour in the adult testis*

Case 8 (Fig 21)	pt aged 30 years

Cases 4 and 8 are identical with cases 1 and 2 of my previous study (38), and case 4 was originally included in *Santesson & Marrubini*'s series (26)

All cases of endodermal sinus tumours of the ovary occur in young patients In *Santesson & Marrubini*'s series (1957) comprising 17 cases, the age ranged from 2½ to 35 years, nine cases occurred in the second decade, four in the third The mean age was 17 9 years In the series of 27 cases reported by *Neubecker & Breen* (1962) the average age was 20 3 years, 85 per cent (23) of the patients being less than 26 years old and 56 per cent (15) were in the age group 16–25-year Regardless of the treatment all cases followed up had a malignant course Case 7 in *Huntingtons et al*'s material, a 7 year-old girl, remained well for more than three years after the operation and is for the time being the only exception As to the endodermal sinus tumours of the infantile testis, the rather favourable outlook—although quite difficult to relate to the histology—has recently been emphasized (16)

SUMMARY AND CONCLUSIONS

1 While testicular tumours belonging to the general category of embryonal carcinomas" form a multiplicity of histologic patterns, which are not sufficiently characterized, the homologous counterpart in the ovary is principally represented by the endodermal sinus tumour (mesoblastoma vitellinum) showing a selective overgrowth of the extra-embryonic mesoblast, intimately associated with yolk sac endoderm suggesting a close embryological relationship between these two layers

2 The typical histologic features vacuolated meshwork, endodermal sinus pattern and primitive vitelline vesicles mimicking transient stages in the embryologic conversion of the primary yolk sac into the secondary sac all express a selective differentiation of yolk sac endoderm in contact with the extra-embryonic mesoderm

3 The interpretation of this vitelline tumour in the human ovary is supported by the recent experimental studies by *Pierce & Dixon*, who during conversion of a transplantable testicular teratocarcinoma of the mouse to the ascites form found a simplification of the tumour pattern contain [illegible]

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[illegible] areas of tumour cells with dense bands of hyalin, which are similar to *Pierce & Dixon*'s finding in experimental tumours in mice The studies of the human ovarian tumours suggest that this hyaline material and the hyalin globules' have a common origin representing a product of secretion of the lining tumour cells

5 The conceptual designation 'embryonal carcinoma', that may be applicable for a complex group of testicular tumours, is not a sufficient

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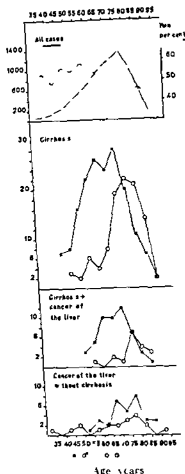
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In 8 cases p c l had been diagnosed during life (biopsy of the liver in 6 of metastases in 2) and in 17 it had been suspected on clinical grounds Other malignant diseases had been suspected in 67 cases

Concomitant diseases site of the tumour in the liver, histological features and the distribution of metastases are given in Tables 2–5 Gross and/or microscopic growth in blood vessels was noted in 82 cases

Acta path et microbiol scandinav 64 430 440 1965

University of Lund Institute of Pathology Malmo Sweden

PRIMARY CARCINOMA OF THE LIVER

A Study of 121 Cases

By

E G H OHLSSON and J G NORDEN

Received 29 i 65

This paper is concerned with the prevalence and some clinical and morphological patterns of primary carcinoma of the liver (p c l) in a Swedish town

MATERIAL AND METHODS

In Malmo a town of about 250 000 inhabitants 99 per cent of patients dying in its *one and only hospital* are autopsied *i e* about 60 per cent of all those dying in Malmo are autopsied at the University Institute of Pathology Routine examination *includes sectioning of the brain* tongue pharynx total gastrointestinal tract biliary tract vertebral column etc and microscopy of all major organs including the prostate and in cases of tumours also of lymph nodes from all major stations in *the trunk* The liver is cut in slices 1 cm thick and sections of at least one piece are studied histologically

The criteria used for the diagnosis of p c l were (a) the absence of co existing tumours that might just as well have been primary and (b) the presence within the tumours of structures resembling those seen in the normal liver Thus 6 cases of undifferentiated tumour of suspected but not proven hepatic origin were rejected (c) intravascular growth and/or extrahepatic metastases In 2 cases malignancy was diagnosed on the basis of cellular atypia only

The requirements for the diagnosis of cirrhosis were diffuseness of the lesion *i e* generalized affection of the liver + regeneration with changed structure (pseudolobuli) + fibrosis

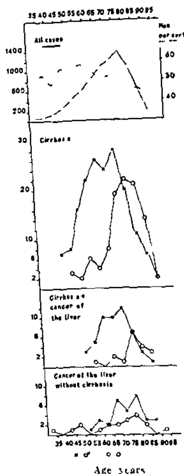
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Statistical methods used chi square and t function The cirrhotic and non cirrhotic groups of p c l were compared with one another and with the total autopsy material 1957 1960 (3 336 subjects above 30 years)

RESULTS

During 7½ years (1957 1964) 8 837 subjects (49 9 per cent males) including 7 795 above 5 years were autopsied Malignant tumours were found in 3,672 (41 6 per cent) Of these 121 (3 3 per cent) proved to be p c l Primary cancer of the liver was ninth in order of frequency and came immediately after cancer of the biliary passages The tumour most commonly encountered was carcinoma of the prostate (mostly occult) followed by carcinoma of the colon and rectum A preliminary report on the unexpected high frequency of p c l was published in 1959 (17)

The incidence, sex and age distribution of cirrhosis alone, of cirrho



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University of Lund, Institute of Pathology, Malmö, Sweden

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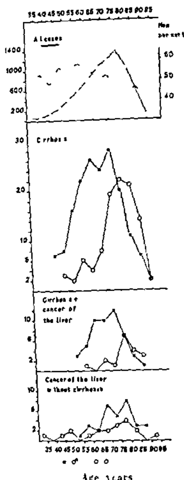
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RESULTS

During 7½ years (1957–1964) 8,837 subjects (49.9 per cent males) including 7,795 above 5 years were autopsied. Malignant tumours were found in 3,672 (41.6 per cent). Of these, 121 (3.3 per cent) proved to be p.c.l. Primary cancer of the liver was ninth in order of frequency and came immediately after cancer of the biliary passages. The tumour most commonly encountered was carcinoma of the prostate (mostly occult) followed by carcinoma of the colon and rectum. A preliminary report on the unexpected high frequency of p.c.l. was published in 1959 (17).

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The incidence, sex and age distribution of cirrhosis alone, of cirrho

TABLE 2

Concomitant Conditions in Primary Carcinoma of the Liver.

	With cirrhosis				Without cirrhosis				P
	Males	Fe-males	Total	Per cent	Males	Fe-males	Total	Per cent	
Total	52	18	70	100	32	19	51	100	
Cholecystolithiasis	12	13	25	40 0	18	11	29	60 8	0 05>P>0 02
Cholecystectomized	1	2	3		1	1	2		
Abuse of alcohol	26	0	26	37 1	6	0	6	11 8	0 01>P>0 001
*Diabetes mellitus**	13	3	16	22 9	4	0	4	7 8	0 05>P>0 02
Syphilis	6	0	6	8 6	0	0	0	0	0 05>P>0 02
Haemochromatosis	2	0	2	1 3	0	0	0	0	
Ulcer or erosion of stomach and or duodenum	11	1	12	22 9	2	2	4	9 8	0 05>P>0 02
Scar after peptic ulcer	2	0	2		0	0	0		
Gastrectomy for ulcer	2	0	2		1	0	1		
Other primary *malignant tumours*§	10	4	14	20 0	4	2	6	11 8	P>0 1
Fatty metamorphosis *of the liver*	33	16	49	70 0	15	12	27	52 9	0 1>P>0 05
slight	29	12	41		11	10	21		
moderate	4	3	7		2	2	4		
severe	0	1	1		2	0	4		
Icterus	26	7	33	47 1	13	4	17	33 3	P>0 1
Ascites	33	12	45	64 3	22	8	30	58 8	
Maelena	15	3	18	25 7	2	3	5	9 8	0 05>P>0 02

* Including the 2 *cases with haemochromatosis, when these are excluded* 0 1>P>0 05

§ 18 cases 1 tumour and 2 had *two tumours (cancer of the stomach in* 3 *colon in* 3, prostate in 6 lung in 5, *kidney in* 1 lip in 1, glioma in 1 Hodgkin s disease in 1

TABLE 3

Site of Primary Carcinoma of the Liver at Macroscopic Examination Judged after Localization of Predominant or most Intensive Growth

	With cirrhosis				Without cirrosis				P
	Males	Fe males	Total	Per cent	Males	Fe ma les	Total	Per cent	
Total	52	18	70	100	32	19	51	100	
Right lobe	22	9	31	44 3	12	7	19	37 3	
Left lobe	4	2	6	8 6	8	4	12	23 5	0 05>P>0 02
Gallbladder region	0	1	1		4	0	4	7 8	
Centrally and caudate lobe	4	0	4		0	1	1		
Diffuse	22	6	28	40 0	8	7	15	29 4	P>0 1
Multiple growths in liver	46	12	58	*82 9*	*27*	*14*	*41*	*80 4*	

TABLE 1

	Number	Age				
		Mean	± s	e	e_D	P
Cirrhosis						
Males	171	66 2	10 3	0 79	1 286	0 001>P
Females	101	75 3	10 2	1 02		
Cancer with cirrhosis						
Males	52	67 6	8 3	1 15	2 158	0 001>P
Females	18	77 4	7 8	1 83		
Cirrhosis without cancer						
Males	119	65 2	12 8	1 18	1 656	0 001>P
Females	83	74 8	10 6	1 17		
Cancer without cirrhosis						
Males	32	72 1	9 2	1 28	3 918	P>0 1
Females	19	66 8	15 5	3 56		
Males above 5 yrs	2060	68 1	14 0	0 10	0 848	0 02>P>0 01
Males cirrhosis	171	66 2	10 3	0 79	0 831	0 001>P
Males, 40–95 yrs	1973	69 7	11 4	0 26		
Females above 5 yrs	2048	70 3	13 9	0 37	1 060	0 001>P
Females, cirrhosis	101	75 3	10 2	1 02	1 044	0 01>P>0 001
Females, 45 95 yrs	1929	72 2	10 8	0 25		
Males above 5 yrs	2060	68 1	14 0	0 10	1 187	
Males cancer + cirrhosis	52	67 6	8 3	1 15	1 169	0 01>P>0 001
Males, 50 90 yrs	1833	70 9	9 8	0 23		
Females above 5 yrs	2048	70 3	13 9	0 37	3 273	0 05>P>0 02
Females cancer + cirrhosis	18	77 4	7 8	1 83	2 285	0 02>P>0 01
Females 55–90 yrs	1328	74 2	9 7	0 27		

DISCUSSION

The highly varying incidence of cirrhosis found in different materials (Denmark 5 9 (10), Finland 3 1 (7), U K 1 0 (18), 2 1 (5), U S A 4 7 (12), 13 9 (16) per cent) may be partly due to differences in sampling methods and in diagnostic criteria In the present autopsy material, which we believe to be fairly representative of a Scandinavian urban population, cirrhosis was demonstrated in 3 1 per cent (3 9 per cent of the males, and 2 3 per cent of the females) or in 3 5 per cent of all subjects above 5 years If incipient and equivocal cases had been included, the frequency would have been 4 5 per cent The values found thus fell well within the limits of the range reported by other Scandinavian workers

The age at death was found to vary significantly with sex in the cirrhotic group, in the cancerous subgroup as well as in the non-cancerous, males dying 9 years earlier then females This may be partly explained by the relatively larger number of alcoholics among the males, this subgroup having a mean age of 63 5 against 70 4 years for other cirrhotic males, among whom a number of unknown alcoholic abusers

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Syphilis	6	0	6	8 6	0	0	0	0	0 05>P>0 02
Haemochromatosis	2	0	2	1 3	0	0	0	0	
Ulcer or erosion of stomach and or duodenum	11	1	12	22 9	2	2	4	9 8	0 05>P>0 02
Scar after peptic ulcer	2	0	2		0	0	0		
Gastrectomy for ulcer	2	0	2		1	0	1		
Other primary malignant tumours§	10	4	14	20 0	4	2	6	11 8	P>0 1
Fatty metamorphosis of the liver	33	16	49	70 0	15	12	27	52 9	0 1>P>0 05
slight	29	12	41		11	10	21		
moderate	4	3	7		2	2	4		
severe	0	1	1		2	0	4		
Icterus	26	7	33	47 1	13	4	17	33 3	P>0 1
Ascites	33	12	45	64 3	22	8	30	58 8	
Maelena	15	3	18	25 7	2	3	5	9 8	0 05>P>0 02

* Including the 2 cases with haemochromatosis, when these are excluded 0 1>P>0 05

§ 18 cases 1 tumour and 2 had two tumours (cancer of the stomach in 3 colon in 3 prostate in 6 lung in 5 kidney in 1 lip in 1 glioma in 1, Hodgkin s disease in 1

TABLE 3

Site of Primary Carcinoma of the Liver at Macroscopic Examination Judged after Localization of Predominant or most Intensive Growth

	With cirrhosis				Without cirrhosis				P
	Males	Females	Total	Per cent	Males	Females	Total	Per cent	
Total	52	18	70	100	32	19	51	100	
Right lobe	22	9	31	44 3	12	7	19	37 3	
Left lobe	4	2	6	8 6	8	4	12	23 5	0 05>P>0 0
Gallbladder region	0	1	1		4	0	4	7 8	
Centrally and caudate lobe	4	0	4		0	1	1		
Diffuse	22	6	28	40 0	8	7	15	29 4	P>0 1
Multiple growths in liver	46	12	58	82 9	27	14	41	80 4	

TABLE 1

	Number	Age				
		Mean	± s	e	e_D	P
Cirrhosis						
Males	171	66 2	10 3	0 79	1 286	0 001>P
Females	101	75 3	10 2	1 02		
Cancer with cirrhosis						
Males	52	67 6	8 3	1 15	2 158	0 001>P
Females	18	77 4	7 8	1 83		
Cirrhosis without cancer						
Males	119	65 2	12 8	1 18	1 656	0 001>P
Females	83	74 8	10 6	1 17		
Cancer without cirrhosis						
Males	32	72 1	9 2	1 28	3 918	P>0 1
Females	19	66 8	15 5	3 56		
Males above 5 yrs	2060	68 1	14 0	0 10	0 848	0 02>P>0 01
Males cirrhosis	171	66 2	10 3	0 79	0 831	0 001>P
Males 40–95 yrs	1973	69 7	11 4	0 26		
Females above 5 yrs	2048	70 3	13 9	0 37	1 060	0 001>P
Females cirrhosis	101	75 3	10 2	1 02	1 044	0 01>P>0 001
Females, 45 95 yrs	1929	72 2	10 8	0 25		
Males above 5 yrs	2060	68 1	14 0	0 10	1 187	
Males cancer + cirrhosis	52	67 6	8 3	1 15	1 169	0 01>P>0 001
Males 50 90 yrs	1833	70 9	9 8	0 23		
Females above 5 yrs	2048	70 3	13 9	0 37	3 273	0 05>P>0 02
Females cancer + cirrhosis	18	77 4	7 8	1 83	2 285	0 02>P>0 01
Females 55 90 yrs	1328	74 2	9 7	0 27		

DISCUSSION

The highly varying incidence of cirrhosis found in different materials (Denmark 5 9 (10), Finland 3 1 (7) U K 1 0 (18) 2 1 (5), U S A 4 7 (12), 13 9 (16) per cent) may be partly due to differences in sampling methods and in diagnostic criteria In the present autopsy material which we believe to be fairly representative of a Scandinavian urban population, cirrhosis was demonstrated in 3 1 per cent (3 9 per cent of the males, and 2 3 per cent of the females) or in 3 5 per cent of all subjects above 5 years If incipient and equivocal cases had been included, the frequency would have been 4 5 per cent The values found thus fell well within the limits of the range reported by other Scandinavian workers

The age at death was found to vary significantly with sex in the cirrhotic group, in the cancerous subgroup as well as in the non cancerous, males dying 9 years earlier then females This may be partly explained by the relatively larger number of alcoholics among the males this subgroup having a mean age of 63 5 against 70 4 years for other cirrhotic males, among whom a number of unknown alcoholic abusers

TABLE 2

Concomitant Conditions in Primary Carcinoma of the Liver

	With cirrhosis				Without cirrhosis				P
	Males	Fe males	Total	Per cent	Males	Fe males	Total	Per cent	
Total	52	18	70	100	32	19	51	100	
Cholecystolithiasis	12	13	25	40 0	18	11	29	60 8	0 05>P>0 02
Cholecystectomized	1	2	3		1	1	2		
Abuse of alcohol	26	0	26	37 1	6	0	6	11 8	0 01>P>0 001
Diabetes mellitus*	13	3	16	22 9	4	0	4	7 8	0 05>P>0 02
Syphilis	6	0	6	8 6	0	0	0	0	0 05>P>0 02
Haemochromatosis	2	0	2	1 3	0	0	0	0	
Ulcer or erosion of stomach and or duodenum	11	1	12	22 9	2	2	4	9 8	0 05>P>0 02
Scar after peptic ulcer	2	0	2		0	0	0		
Gastrectomy for ulcer	2	0	2		1	0	1		
Other primary malignant tumours§	10	4	14	20 0	4	2	6	11 8	P>0 1
Fatty metamorphosis of the liver	33	16	49	70 0	15	12	27	52 9	0 1>P>0 05
slight	29	12	41		11	10	21		
moderate	4	3	7		2	2	4		
severe	0	1	1		2	0	4		
Icterus	26	7	33	47 1	13	4	17	33 3	P>0 1
Ascites	33	12	45	64 3	22	8	30	58 8	
Maelena	15	3	18	25 7	2	3	5	9 8	0 05>P>0 02

* Including the 2 cases with haemochromatosis, when these are excluded 0 1>P>0 05

§ 18 cases 1 tumour and 2 had two tumours (cancer of the stomach in 3, colon in 3, prostate in 6, lung in 5 kidney in 1, lip in 1, glioma in 1, Hodgkin s disease in 1

TABLE 3

Site of Primary Carcinoma of the Liver at Macroscopic Examination Judged after Localization of Predominant or most Intensive Growth

	With cirrhosis				Without cirrcosis				P
	Males	Fe males	Total	Per cent	Males	Fe ma les	Total	Per cent	
Total	52	18	70	100	32	19	51	100	
Right lobe	22	9	31	44 3	12	7	19	37 3	
Left lobe	4	2	6	8 6	8	4	12	23 5	0 05>P>0 02
Gallbladder region	0	1	1		4	0	4	7.8	
Centrally and caudate lobe	4	0	4		0	1	1		
Diffuse	22	6	28	40 0	8	7	15	29 4	P>0 1
Multiple growths in liver	46	12	58	82 9	27	14	41	80 4	

TABLE 4

Histological Appearance of Primary Carcinoma of the Liver

	With cirrhosis				Without cirrhosis				P
	Males	Females	Total	Per cent	Males	Females	Total	Per cent	
Total	52	18	70	100	32	19	51	100	
Hepatocellular at least partly	52	18	70	100	31	19	50	98	
Highly differentiated	13	5	18	25 7	1	3	4	7 8	
Moderately differentiated	36	13	49	70 0	29	16	45	88 2	
Slightly differentiated	3	0	3	4 3	1	0	1	2	
Growing in rings	26	6	32	45 7	10	6	16	31 4	P>0 1
Purely cholangiocellular	0	0	0		1	0	1	2	0 02>P>0 01
Hepatocellular + cholangiocellular	5	1	6	8 6	6	6	12	23 5	
Partly polymorphus	25	10	35	50 0	14	10	24	47 1	
Partly undifferentiated	17	6	23	32 9	15	8	23	45 1	
Fatty tumour cells	19	9	28	40 0	4	2	6	11 8	0 001>P
Bile formation in tumour	21	8	29	41 4	10	5	15	29 4	P>0 1
Growth in blood vessels	37	9	46	65 7	22	12	34	66 7	

may further influence the mean The mean age at death of the 156 male alcoholics without cirrhosis was 61 5 years *Garceau et al* (9), however, found the age at death of patients with cirrhosis to be higher among alcoholics

The average age at death of males as well as of females with cirrhosis *cum* p c l was about 2½ years higher than the corresponding values for cirrhosis without cancer, but P>0 1 and no conclusion can be drawn about the interval between the onset of the tumours and death

The 121 cases of p c l found at 8 837 unselected autopsies during 7½ years (1 37 per cent of total, 3 3 per cent of all cases of malignant tumour) means a relatively higher frequency than that found in other materials, most of which do not represent a well defined population or are sampled during longer periods (cf *e g* survey by *Ervasti* (7)) The finding of p c l in 70 of 272 cases of cirrhosis (25 7 per cent, 30 4 in males, 17 8 per cent in females) is also a relatively high number

Despite the high frequency of cancer in the present cases of cirrhosis especially when compared with European and American figures, the incidence of cirrhosis in the 121 cases of cancer (61 9 per cent in males,

TABLE 5

	With cirrhosis				Without cirrhosis				P
	Males	Females	Total	Per cent	Males	Females	Total	Per cent	
Total	52	18	70	100	32	19	51	100	
Extrahepatic metastases	30	8	38	54.3	26	16	42	82.4	0.01>P>0.001
Regional lymph nodes	18	5	23	32.9	15	13	28	54.9	0.02>P>0.01
Para aortic lymph nodes	11	4	15	21.4	13	10	23	45.1	0.01>P>0.001
Mediastinal lymph nodes	10	3	13	18.5	6	8	14	27.5	P>0.1
Supraclavicular lymph nodes	4	2	6	8.8	4	5	9	17.6	P>0.1
Inguinal lymph nodes	2	0	2	2.9	3	1	4	7.8	
Peritoneum	5	1	6	8.6	10	5	15	29.4	0.01>P>0.001
Spleen	2	0	2	2.9	2	1	3	5.9	
Vertebral column	8	1	9	13.0	6	2	8	15.7	
Other bones	7	0	7	10.0	4	1	5	9.8	
Lungs	19	5	24	34.3	17	8	25	49.0	P>0.1
Pleurae	7	1	8	11.4	6	1	7	13.7	
Heart	1	0	1		0	0	0		
Adrenals	4	1	5	7.1	8	5	13	25.5	0.01>P>0.001
Kidneys	2	0	2	2.9	3	2	5	9.8	
Prostate	0	0	0		1	0	1		
Testes	0	0	0		1	0	1		
Ovaries	0	1	1		0	0	0		
Thyroid	1	0	1		0	0	0		
Stomach	2	0	2	2.9	1	0	1		
Continuous growth into									
Gallbladder	4	1	5	7.1	5	3	8	15.7	P>0.1
Diaphragm	3	0	3	4.3	5	0	5	9.8	
V. porta	13	1	14	20.0	2	1	3	5.9	0.02>P>0.01
V. hepatica	5	1	6	8.6	2	2	4	7.8	

48.6 per cent in females) was about the same as the mean figures given for Europe and North America (13).

A history of previous hepatitis was known only in one case. Malaria had been noted in two cases, one of which included also a history of yellow fever. Of the 6 subjects with a history of syphilis, 3 had also abused alcohol. As to the difference in abuse of alcohol between the cirrhotic and the non-cirrhotic group, it is not known whether, or to what extent, the diagnosis of cirrhosis had intensified the inquiry into such abuse. The material based on police records alone was not biased in this respect though certainly in others, and showed alcoholic abuse in 15 cases (28.8 per cent) among the 52 males with cirrhosis *cum* p.c.l. against 16 (17.4 per cent) out of 92 cases of cirrhosis in the general material. This difference, though P>0.1, argues, if anything,



Figs 1 6

Fig 1 Hepatocellular cancer rings with bile drops × 75
Fig 2 Mixed hepato and cholangio cellular cancer × 75
Fig 3 Solid cords of hepatocellular cancer × 75
Fig 4 Region with cholangiocellular cancer × 75
Figs 5 and 6 Hepato cellular cancer with varying degree of fatty vacuolization × 75



Figs 7 12

Figs 7 an ...lization × 75
Fig 9 × 120
Fig 10 cancer × 75
Fig 11
Fig 12 Growth in blood vessel × 75



I gs 1 6

Fig 1 1 1 n r r n with l le lrops × 75
Fig 2 can er × 75
F g 3 × 6
Fig 4 Regio r × 5
I igs 5 ar 1 6 Hepato cellular cancer w th varying d gr e f fatty vacuolization × 5

mortality among cirrhotic patients before metastases have had time to (T ? n co ith backward growth of hepato cellular cancer
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SUMMARY

During 7½ years 121 cases of primary carcinoma of the liver were found at 8 837 autopsies covering approximately 60 per cent of all deaths in Malmo In 272 cases of cirrhosis as well as in the subgroups cirrhosis *with cancer* and *cirrhosis without cancer* the duration of life of the males was significantly shorter than that of the females A high incidence (25 7 per cent) of cancer was found in these cirrhotic livers Comparison is made between 70 cases with cancer and cirrhosis and 51 cases with cancer without cirrhosis Abuse of alcohol was more common in the cirrhosis + cancer group and also somewhat more common than in cirrhosis without cancer Diabetes mellitus, peptic ulcers and fatty tumour cells were also found more frequently among patients with cirrhosis + cancer, cholecystolithiasis cholecystectomy more frequent ly *in the non cirrhotic cancer group, in which the cholangio cellular* type of tumour tended to be more common though usually mixed with hepato cellular type Metastasis was more common in the non cirrhotic group

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against the conception that patients with dietary cirrhosis are less liable to develop p c l than patients with other types of cirrhosis Of 1698 males above 20 years in the general autopsy material, 176 (11 4 per cent; 9 6 per cent among those above 50 years) were known from the police records to be alcoholics

Diabetes mellitus was known in 13 (25 per cent) out of 52 males with cirrhosis *cum* p c l, which means a significantly (0 001>P) higher frequency than in the entire autopsy material, where diabetes in males above 50 years was found in 85 of the 1390 (6 1 per cent) The degree of significance was the same when the two cases of haemochromatosis were excluded

It was originally suspected that cancer in non cirrhotic livers developed from a focal cirrhosis But only in two cases did the findings possibly support such a conjecture

Histologically the hepatocellular type of p c l was predominant in some parts of the tumours Only one case of a purely cholangio-cellular type was found Some cases were of mixed hepato- and cholangio cellular type Care was taken not to confuse proliferating non-neoplastic ductules in a fibrous stroma with cholangio cellular cancer In hepato cellular cancer the cells may be arranged in rings, which often contain drops of bile (Fig 1) These structures, which are very useful for the diagnosis of cancer, should not be mistaken for the cholangio-cellular type of cancer

In some areas the picture suggests a gradual transition between cholangio-cellular-tubular structures, solid cords of small cells and hepato-cellular forms This favours the view, advocated by *Doljanski & Roulet* (3) but challenged by others (11, 21), that the cholangio cellular epithelium can change into hepato-cellular epithelium and *vice versa* In some series mixed types are common, but in most of the series they are few or absent In our material the cholangio-cellular type was more common in the non-cirhotic group This was probably because of the regenerating stimulus in cirrhosis which tends to cause the hepatic cells to change into differentiated cancer cells of hepatic cell type rather than to form ductules Another possibility is that in the absence of liver cirrhosis changes in the biliary tract, *e g* in biliary stasis often including ductular proliferation, might be a more important factor here than in the cirrhotic group In fact, cholecystolithiasis tended to be more common in the non-cirrhotic group When compared with that part of the entire autopsy material above 30 years, the non-cirrhotic p c l group showed a higher incidence (0 01>P>0 001) of cholecystolithiasis or cholecystectomy The cirrhotic p c l group showed no such increased incidence It should be mentioned that out of 5 subjects with cancer in the vicinity of the gallbladder, 4 had also cholecystolithiasis

Metastases were significantly more common in the non-cirrhotic group This cannot be explained by any histological differences in cell types or tendency to growth in blood vessels but possibly by the higher

mortality among cirrhotic patients before metastases have had time to develop The 3 cases with backward growth of hepato-cellular cancer in the portal vein into the stomach deserve special attention Such growth may both clinically and macroscopically simulate cancer of the stomach with metastases of the liver

SUMMARY

During 7½ years 121 cases of primary carcinoma of the liver were found at 8837 autopsies covering approximately 60 per cent of all deaths in Malmo In 272 cases of cirrhosis as well as in the subgroups cirrhosis with cancer and cirrhosis without cancer the duration of life of the males was significantly shorter than that of the females A high incidence (25 7 per cent) of cancer was found in these cirrhotic livers Comparison is made between 70 cases with cancer and cirrhosis and 51 cases with cancer without cirrhosis Abuse of alcohol was more common in the cirrhosis + cancer group and also somewhat more common than in cirrhosis without cancer Diabetes mellitus, peptic ulcers and fatty tumour cells were also found more frequently among patients with cirrhosis + cancer cholecystolithiasis cholecystectomy more frequently in the non cirrhotic cancer group in which the cholangio cellular type of tumour tended to be more common though usually mixed with hepato cellular type Metastasis was more common in the non cirrhotic group

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HISTOCHEMISTRY OF RAT THYMUS DURING INVOLUTION INDUCED BY ALKYLATING AGENTS

By

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E O KARAHARJU

Received 4 ii 65

A number of authors have studied the histological changes which occur during thymus involution induced by x rays (*Murray* 1948, *Schreck* 1948, *Trowell* 1952) various cytostatics (*Pappenheimer et al* 1920, *Kindred* 1947), exogenic stress (*Andreasen* 1943), and certain steroid hormones (*Ringertz et al* 1952, *Toro* 1961, *Gyllensten* 1962) Most of these investigations have been concerned with changes in the weight and the relative number of different cell types in the gland Although nuclear changes and an inhibition of mitosis have been observed during the degeneration of the thymus (*Fisher et al* 1959, *Hagen* 1961 *Scaife et al* 1961), no changes have been seen in the nucleic-protein metabolism during the involution process Consequently, the way in which alcylating substances inhibit nucleic acid synthesis still remains to be explained

However histochemical studies have been made on the metabolic alterations which occur in the thymus after x-ray irradiation (*Smith et al* 1953 *Smith* 1956, *Smith* 1962) Biochemical determination of the activity of different enzymes of the whole organ has been made during involution (*Petersen et al 1952*, *Dubois et al* 1954, *Dougherty et al* 1960) These studies on thymus involution have given rise to an interesting problem differences exist in the changes which occur in the medulla and cortex of the thymus following thymolytic action (*Trowell* 1961) The present investigations was undertaken with a view to further examination of this different response of enzyme activity of the medulla and the cortex to a thymolytic agent The activity of acid phosphatase succinate and NADH-tetrazolium reductase was studied histochemically following the involution of rat thymus by cyclophosphamide

MATERIALS AND METHODS

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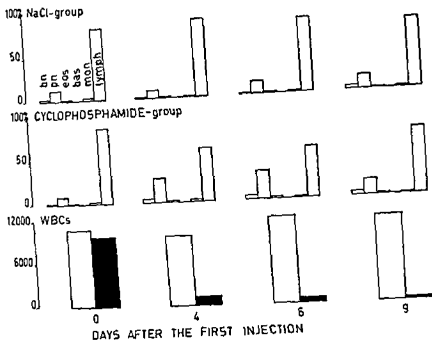


Fig 1

after the first cyclophosphamide injection and further treatment brought about a progressive decrease The most marked effect on the blood leucocytes of the rat was apparent just before decapitation No significant changes were observed in the differential count

Histological changes—In the thymus of the cyclophosphamide-injected rats, almost no lymphocytes were found in the cortex, and only a few pyknotic cells and some macrophages The lymphocytes seemed almost undisturbed in the medulla, although a slight numerical decrease was apparent after treatment with the cytostatic, correspondingly, the medulla stained much darker with haematoxylin than the cortex The reticulum cells appeared to be the only surviving cortex cells after cyclophosphamide treatment (Figs 2 and 3) The thymuses of the control rats displayed no deviation from the normal histological picture

Histochemical changes—1 *Lipids* The thymus of animals treated with cyclophosphamide showed numerous lipid droplets in the cortex, not visible in the thymus of control rats (Figs 4 and 5)

2 *Succinate tetrazolium reductase* Histochemically demonstrable succinate tetrazolium reductase activity in normal rat thymus is rather weak Only a few cells in the corticomedullary junction displayed enzyme activity (Fig 6) Following treatment with cyclophosphamide, the

dose being 150 mg The control group consisted of 21 animals, into which 0 5 ml of isotonic sodium chloride was injected intraperitoneally in a corresponding manner Standard diets were used, and water was provided ad libitum for all the animals

The rats were weighed every second day and at the end of the experiment The animals were killed by decapitation, and the thymus, the spleen and the adrenals were weighed

The total number of blood leucocytes was counted from lingual vein blood on the first fourth sixth, and tenth days of the experiment For a differential leucocyte count, blood films were prepared from each sample, and 200 leucocytes were classified on each slide

For histological examination, pieces of the thymus spleen, and iliac lymphnodes were fixed in sublimate-formol for one day Sections of paraffin embedded tissue were cut at 4 μ, and stained with haematoxylin eosin, and by application of the periodic acid Schiff-technique (*McManus* 1946)

In addition, pieces of the thymus were immediately frozen with dry ice, and sections cut at 20 μ with a cold microtome (cryostat) The following reactions were determined

1 Staining of lipids with Oil Red O in 70 per cent isopropanol (*Pearse* 1961)
2 Tetrazolium reductase activity, using reduced nicotinamide adenine dinucleotide (NADH) or succinate as substrates (*Nachlas et al* 1957, 1958)
3 Acid phosphatase (*Gomori* 1941)

RESULTS

The weight of the body and of various organs are presented in Table 1

Weight response —Animals injected with cyclophosphamide failed to grow, whereas there was a normal increase in the weight of normal animals and those injected with sodium chloride during the course of the experiment The weight of the thymus and spleen had decreased markedly after cyclophosphamide treatment, but there was discernible a slight increase in the weight of the adrenals of these animals

TABLE 1

The Response to Injections of Cyclophosphamide of the Weights Thymuses Spleens and Adrenals of Rats

	Cyclophosphamide group I (7)		Cyclophosphamide group II (10)		NaCL group III (9)		Kontrol group IV (4)	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Weights of spleens mg	195 1	± 7 4	207 7	± 4 9	668 0	± 75 0	553 0	± 40 9
Weights of adrenals mg	64 5	± 2 9	60 1	+ 4 6	53 6	± 2 0	50 8	± 3 3
Weight of thymus mg	56 1	+ 3 8	68 1	± 8 7	318 3	± 14 6	380 0	± 42 5
Increase in mean weight of rats during experiments g	— 3 4		+ 4 1		+ 17 5		+ 29 2	

Blood counts —Fig 1 shows the effect of cyclophosphamide on blood leucocytes The total number of leucocytes per cu mm had fallen even

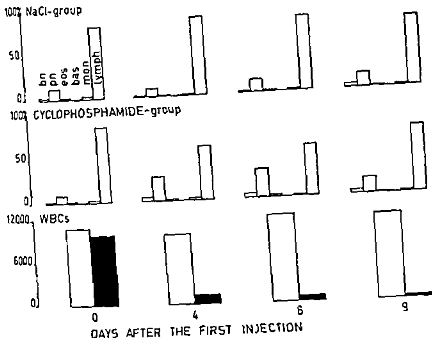


Fig 1

Effects of cyclophosphamide on rat blood leucocytes The total number of leucocytes per cu mm (WBCs) diminshed even after the first cyclophosphamide injection No significant changes were observable in differential counts

after the first cyclophosphamide injection and further treatment brought about a progressive decrease The most marked effect on the blood leucocytes of the rat was apparent just before decapitation No significant changes were observed in the differential count

Histological changes.—In the thymus of the cyclophosphamide-injected rats almost no lymphocytes were found in the cortex, and only a few pyknotic cells and some macrophages The lymphocytes seemed almost undisturbed in the medulla, although a slight numerical decrease was apparent after treatment with the cytostatic, correspondingly, the medulla stained much darker with haematoxylin than the cortex The reticulum cells appeared to be the only surviving cortex cells after cyclophosphamide treatment (Figs 2 and 3) The thymuses of the control rats displayed no deviation from the normal histological picture

Histochemical changes.—1 *Lipids*. The thymus of animals treated with cyclophosphamide showed numerous lipid droplets in the cortex, not visible in the thymus of control rats (Figs 4 and 5)

2 *Succinate tetrazolium reductase* Histochemically demonstrable succinate tetrazolium reductase activity in normal rat thymus is rather weak Only a few cells in the corticomedullary junction displayed enzyme activity (Fig 6) Following treatment with cyclophosphamide, the



Figs 2–3

Fig 2 Cyclophosphamide treated rat thymus The thymus inversion is very clear Note the almost total lymphocytolyse in the cortex (M = medulla C = cortex) × 500

Fig 3 Normal rat thymus The cortex is densely packed with dark stained lymphocyte × 500 (Figs 1 and 2 Haematoxylin Eosin staining)

succinate tetrazolium reductase activity in the cortex was greatly increased Numerous strongly positive large cells could be seen in the cortex, dyed purple with Nitro-BT (Fig 7) No change was evident in the enzyme activity in the medulla

3 *NADH-tetrazolium reductase* Rather scanty NADH-tetrazolium reductase activity is apparent in normal rat thymus, it is visible only around the blood vessels, and also to a slight extent in the epithelial reticulum cells and lymphocytes in the cortex and medulla (Fig 8) After cyclophosphamide injections, a marked increase in NADH tetra-

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Figs 4 11

Fig 4 *Untreated rat thymus Only a few cells are stained in the cortico medullary* junction these have rich lipid droplets × 130 (Figs 4 and 5 Oilred O)

Fig 5 After cyclophosphamide injections a large number of lipids accumulate in the cortex The medulla is displaying more marked staining × 130

Fig 6 Succinate tetrazolium reductase activity in the normal rat thymus A few large cells in the cortico medullary junction are stained positive with Nitro BT × 130

Fig 7 After cyclophosphamide treatment in the cortex there are observable numerous strongly formazan positive cells No changes are apparent in the medulla × 130

Fig 8 NADH tetrazolium reductase activity in the normal rat thymus is weak boundary

Fig 9 trazolium reductase activity is discernible in the cortex very distinctly restricted to almost negative medulla × 130

Fig 10 Gomori s lead sulphide precipitates in untreated rat thymus can be seen in the few cells in the cortico medullary junction The cortex and medulla are almost negative × 130

Fig 11 After cyclophosphamide injections very marked acid phosphatase activity is visible in the cortex and the medulla remains unchanged × 130

Figs 4–11 Frozen sections cut at 20 µ


C
M
4
C
M
5
C
M
6
C
M
7
M
C
C
8
M
9
M
C
10
C
M
11

zolium reductase activity could be seen in the cortical cells, but no change of activity in the medulla (Fig 9) Apparently the epithelial reticulum cells were the site of the increased NADH-tetrazolium reductase activity.

4 *Acid phosphatase* Acid phosphatase activity in the normal rat thymus borders the cells in the cortico-medullary junction, and some few cells in the medulla No activity has been found in the cortex of the rat thymus (Fig 10) After cyclophosphamide injections, strong acid phosphatase activity appeared in the cortex of the thymus, sharply restricted to the medulla, where no activity was discernible (Fig 11)

5 *PAS-positive materials* PAS-positive material was found in normal thymus cells at the cortico-medullary junction, and in mast cells in connective tissue septa Following cyclophosphamide injections, a slight increase in PAS-positive substances was noted in the cortex

DISCUSSION

The present investigation has demonstrated a histochemically apparent difference in the cortical and medullary cells of the rat thymus following involution induced by cyclophosphamide a significant increase in the activity of tetrazolium reductases and acid phosphatase occurred in the cells of the cortex

To date, the various published reports of investigations of thymus involution have in general proved a rise in the activity of many cellular enzymes, and also during involution caused by different agents Nevertheless, there have been some contradictory findings Most of these observations are confined to a consideration of the total activity of enzymes of the organ, disregarding the localization of the enzyme activity in various cells and in different parts of the thymus

According to *Smith* (1956), the activity of acid phosphatase of the thymus cortex increases during x-ray induced involution, as shown by histochemical determination, and a large amount of lipids appears in the cortex during the degeneration phase (*Smith et al* 1953) In involution induced by x-irradiation a decrease in succinic dehydrogenase activity has been found (*Smith* 1962), but an increase in ATP-ase activity especially in the cortex Nevertheless, the GP-ase and A-5 P-ase activity of mouse thymus is not significantly altered during the course of involution

An elevated acid phosphatase activity (*Rahman* 1962), and an increase in adenosintriphosphatase and 5-nucleotidase (*Dubois et al* 1954) have been demonstrated biochemically in the rat thymus following x-irradiation Further, it is known that the activity of other enzymes increases during involution An increase in β-glucuronidase activity has been shown by *Pellegrino et al* (1956) in the rat thymus during involution induced by fasting and by *Pellegrino et al* (1957) and *Rahman* (1962) following x irradiation *Sachs et al* (1962) have, by

means of involution induced by cortisone, observed an increase in *β glucuronidase, katepsin and arylsulfatase*

According to *De Duve* (1959), some lysosomal enzymes display characteristic changes under conditions in which an involution and a degeneration of tissue occur Enzymes are liberated in the cytoplasm, and simultaneously, there is a retention of enzymes when the tissue loses nitrogen

The dose of cyclophosphamide used in the experiments led to an almost complete thymolytic reaction Almost all the lymphocytes of the cortex disappeared, whereas a number of lymphocytes survived in the medulla *Regaud et al* (1912), *Tshassownikov* (1929), and *Murray* (1948) have shown that during thymus involution by x-irradiation, the surviving lymphocytes in the medulla outnumber those of the cortex, a phenomenon known as thymus "inversion" In addition to this histological thymus "inversion" in our experiments, the still existing corticomedullary boundary was histochemically evident, by reason of the strong enzyme activity in the cortex cells The alterations in NADH-tetrazolium reductase activity during the course of involution are of special interest An intensive increase of NADH-tetrazolium reductase activity in the epithelial reticulum cells of the cortex was observable during the involution of thymus following cyclophosphamide injections, and a very sharp boundary existed between the cortex and medulla

Auerbach (1961) has demonstrated that the lymphocytes of thymus originate in the embryonic thymus rudiment in the epithelial reticulum cells, and *Miller* (1961) has established that the thymus is essential to a proper development of the lymphoid structures throughout the body

The high lymphocyte content of thymus cortex results in a more extensive involutionary effect in the cortex than in the medulla In *Trowell's* (1961) experiments the sensitivity of cortex lymphocytes to x-irradiation was four times that of medullary lymphocytes It is probable that the metabolic changes in the thymus during involution occur principally in the cortex The lymphocytolysis in the thymus cortex is followed by a histochemically demonstrable rise in the activity of succinate tetrazolium reductase NADH-tetrazolium reductase and acid phosphatase These facts seem to indicate that the cortex may play the major role in the production of lymphocytes in the thymus

The metabolic rise in enzyme activity may, as regards lysosomal acid phosphatase arise from the involution and the degenerative changes Conversely, the rise in the microsomal NADH-tetrazolium reductase activity may be connected with the disturbed function of the epithelial reticulum cells of the cortex

SUMMARY

A high total dose of cyclophosphamide was given intraperitoneally to 30 rats during a period of 10 days The growth of the animals ceased, and an almost complete thymolytic reaction occurred, displaying the histological picture of thymus "inversion"

A histochemical study of the animals injected with cyclophosphamide and those used as controls, demonstrated a clearly raised activity of succinate and NADH tetrazolium reductases and acid phosphatase in the cortex of the thymus during involution following cyclophosphamide treatment Furthermore, a slight increase in PAS positive material and the occurrence of lipid droplets was noted in the cortex The enzyme activities in the medulla were unaltered The demarcation between the cortex and the medulla in the thymus of treated animals, still distinct in the histological picture, was also histochemically distinct and clear, the raised enzyme activity being strictly limited to cortical cells

These results seem to indicate that the thymus cortex and the medulla are tissues of different metabolic and functional character

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in 2 cases These 14 cases are therefore of little value in the investigation of the possible role played by oxygen treatment Of the remaining 14 subjects, 11 had been treated with oxygen of varying concentration The highest concentration given was at least 50 per cent in 7 and 40–49 per cent in 4 In none of these 11 cases was it below 40 per cent (Table 1)

TABLE 1

Distribution of Patients with Hyaline Membranes According to Concentration of Oxygen in Inspired Gases

Concentration of oxygen	Entire material	Hyaline membranes in patients with and without pulmonary infection uraemia radiation reactions	
		with	without
0 extra O_2 (group I)	19	1	1
21–29 % O_2 (group II)	84	9	2
40–49 % O_2 (group III)	28	4	4 (14 %)
> 49 % O_2 (group IV)	19	0	7 (37 %)
Total	150	14	14

In 2 of the remaining 3 cases the amount of oxygen supplied is not known with certainty, and in the third no extra oxygen was given One of these two subjects had had pulmonary cancer, and, though the patient had not been tracheotomized, the oxygen had probably been administered via a nasal catheter The hyaline membranes were only scanty The other patient died in a picture of status asthmaticus during tracheotomy and had presumably been given brief oxygen treatment via a tracheal tube Changes resembling pneumonic foci and containing hyaline membranes were found in one region of the left lower lobe Culture of material from the foci gave no growth The third patient was a 32 year old woman who had sustained widespread intracranial haemorrhage She was hyperventilated in a respirator (in an attempt to suppress the intracranial hypertension) Autopsy revealed signs of aspiration of blood from the tracheostoma, which may explain the pulmonary hyaline membranes

The 11 subjects who had been treated with oxygen and developed hyaline membranes were distributed on 3 groups Those with small and local changes were assigned to the first group those with moderate changes to the second group and those with pronounced changes in practically all of the many sections studied in each case, to the third group Table 2 gives the severity of the pulmonary changes in the individual cases together with the highest concentration of oxygen supplied and the duration of treatment in the respirator The duration of treatment seems to be of importance In the 3 cases with the most widespread changes treatment had been given for 5, 13 and 2 days, while in those with less pronounced changes the shortest duration of treatment was 5 hours The following case histories of the 3 subjects with

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OXYGEN TREATMENT AND HYALINE PULMONARY MEMBRANES IN ADULTS

By

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Received 4 ii 65

Pulmonary hyaline membranes in newborns and the possible causal rôle played by oxygen treatment have received much space in the literature Much less attention has been given to this condition in adults Such membranes have been found in association with various diseases (uraemia, radiation reactions, pulmonary infections), but the possible rôle played by oxygen treatment has not been discussed to the same extent

This paper is concerned mainly with the occurrence of pulmonary hyaline membranes found at autopsy of subjects previously treated with oxygen at a respiratory centre

MATERIAL AND METHODS

The material consisted of an autopsy series from the respiratory centre of Malmö general hospital It covers the years 1960–1963 Of all the patients who had been treated with oxygen and died 98 per cent were autopsied Seven of the subjects were excluded because of lack of historical data or incomplete histological examination

The primary material consisted of 150 subjects including 131 who had been treated with oxygen The respirator used was a volume controlled apparatus (Lundia) The oxygen concentration of the inspired gases was thus known The material was divided into groups according to the highest concentration of the oxygen in the inspired gases (Table 1) All cases in which oxygen had been given via a nasal catheter were assigned to group II This group also included a few cases in which the concentration of the oxygen in the inspired gases was not known The period of treatment in these cases was as a rule short e g in association with tracheotomy Some of the patients had also been subjected to other operations shortly before death, which thus increased the total amount of oxygen given

Histological examination of the lungs always included at least one section though often several of each lobe The sections were stained with haematoxylin eosin In selected cases with hyaline membranes the examination included staining according to van Gieson and MacManus as well as Mallory s method for fibrin

RESULTS

Hyaline membranes were found in 28 of 150 subjects examined (Table 1) In 14 of these cases the membranes could plausibly be ascribed to factors other than treatment with oxygen, *viz* pulmonary infections (in 12, including 2 with coexisting uraemia), and radiation

in 2 cases These 14 cases are therefore of little value in the investigation of the possible role played by oxygen treatment Of the remaining 14 subjects, 11 had been treated with oxygen of varying concentration The highest concentration given was at least 50 per cent in 7 and 40–49 per cent in 4 In none of these 11 cases was it below 40 per cent (Table 1)

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the most pronounced pulmonary changes may serve as illustrative examples of the clinical picture

Severity of " TABLE 2 *ration*

Pat	Diagnoses	Hyaline membranes	Highest concentration and duration of O	Total duration of O_2 treatment
459/60	Bronchitis chron + Emphysema pulm	Scanty	50 % 8 hr	12 days
457/61	Stenosis valv mitral + Cor incomp	Scanty	45 %–18 hr	18 hr
484/61	Cancer cardiae op + Sepsis	Scanty	53 %–1 day	9½ days
433/62	Infarctus cordis + Asthma bronchiale	Scanty	47 % 5 hr	14 hr
713/62	Fractura tibiae + Embolia pulm	Scanty	80 %–3/4 hr	4 hr
1325/63	Combustio + Sepsis	Scanty	40 %–1 day	1 day
455/60	Ulcus ventriculi op + Sepsis	Moderate	55 %–2 hr	1 day
1141/62	Carcinoma bronchiale op	Moderate	50 % 5 hr	5 hr
640/62	Emphysema pulm + Haemopneumothorax	Widespread	50 % 4 5 hr	5 days
291/63	Ulcus pylori op	Widespread	40 % 6 days	13 days
340/63	Vitium organicum cordis op	Widespread	70 % 5 hr	2 days

A S 640/62) A female aged 18 who had previously felt well suddenly got symptoms suggesting spontaneous pneumothorax Chest x ray showed condensation of almost the entire right lung and 400 ml of a sanguinolent fluid was removed by thoracocentesis Coagulation studies of the blood revealed nothing abnormal The patient was given blood transfusions and treatment with pleural suction and the atelectasis disappeared The patient was also treated with antibiotics and corticosteroids Owing to unsatisfactory ventilation tracheotomy was done and the patient was placed in a respirator Despite controlled inspiration of gases with a high concentration of oxygen the patient remained hypoxaemic Celocurin was given besides which the patient was treated with hypothermia but without any demonstrable effect the hypoxia progressed and the patient died one week after the onset Autopsy revealed small fibrous scars and emphysema bullae in the apex of each lung as well as right sided haemopneumothorax Practically all of the histological sections of the lungs showed widespread fibrinous exudate in the pulmonary alveoli which in most areas were linked by hyaline menbranes In the frequently oedematous septa the capillaries were congested The former showed strikingly numerous alveolar phagocytes everywhere but only few leucocytes and plasma cells

Oxygen treatment 35–40 per cent oxygen mixture for 18 hours 45 47 per cent for 4 days and 50 per cent for 4–5 hours

E L (291/63) A woman aged 64 operated u[illegible] pyloric ulcer She was reoperated upon 10 days [illegible] intraperitoneal abscess Culture of the blood gave [illegible] Three weeks after the first operation the wound ruptured and was resutured The postoperative course was comp[illegible] [illegible]nt was trachetomized and placed in a [illegible] later however the patient had an att[illegible] sternal heartmassage Later, respiration again became impaired and bronchoscopy showed the bronchi to be covered with a thick crust The patient died about 5 weeks after



Figs 1 2

Fig 1 (455 60) Hyaline membranes on walls of alveoli Numerous alveolar phagocytes Haematoxylin eosin × 340

Fig 2 (640 62) Pulmonary alveoli show extensive fibrinous exudate with striking hyaline membranes Septa oedematous Numerous alveolar phagocytes Mc Manus × 120

the most pronounced pulmonary changes may serve as illustrative examples of the clinical picture

TABLE 2

Severity of Hyaline Membranes Highest Concentration of Oxygen Used and Duration of Oxygen Treatment in 11 Patients Treated with Oxygen

Pat	Diagnoses	Hyaline *membranes*	Highest concentration and duration of O	Total duration of O treatment
459/60	Bronchitis chron + Emphysema pulm	Scanty	*50 %–8 hr*	*12 days*
457/61	Stenosis valv mitral + Cor incomp	Scanty	45 %–18 hr	18 hr
484/61	Cancer cardiae op + Sepsis	Scanty	53 %–1 day	9½ days
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Oxygen treatment 35–40 per cent oxygen mixture for 18 hours 45 47 per cent for 4 days and 50 per cent for 4–5 hours

E L (291/63) A woman aged 64 operated upon with closure of a perforating pyloric ulcer She was reoperated upon 10 days later because of re perforation and intraperitoneal abscess Culture of the blood gave growth of *Staphylococcus aureus* The[illegible] Three weeks after the first operation the [illegible] postoperative course was complicated by re[illegible] *trachetomized and placed in a respirator wi*[illegible] however the patient had an attack of heart [illegible] heartmassage Later respiration again became impaired and bronchoscopy showed the bronchi to be covered with a thick crust The patient died about 5 weeks after

after ..ya the memb ..ucs. ..

Oxygen treatment 33 per cent oxygen mixture for 7 days then 40 per cent for 6 days

S.L. (310 63) An 18 year old man who had been operated upon for tetralogy of Fallot with left sided anastomosis according to Blalock, which was now regarded as obliterated Re-operation with extracorporeal circulation and insertion of a teflon prosthesis at the s te of the septum and excision of the subvalvular stenosed segment

partly oedematous septa and some phagocytes in the alveoli In some areas the alveoli contained eosinophilic oedematous fluid In some areas small bronchopneumonia were seen around the bronchi

Oxygen treatment (in addition to that at operation) about 30 per cent oxygen m xture for 12 hours 40–53 per cent f r about 24 hours and 70 per cent for 5 hours

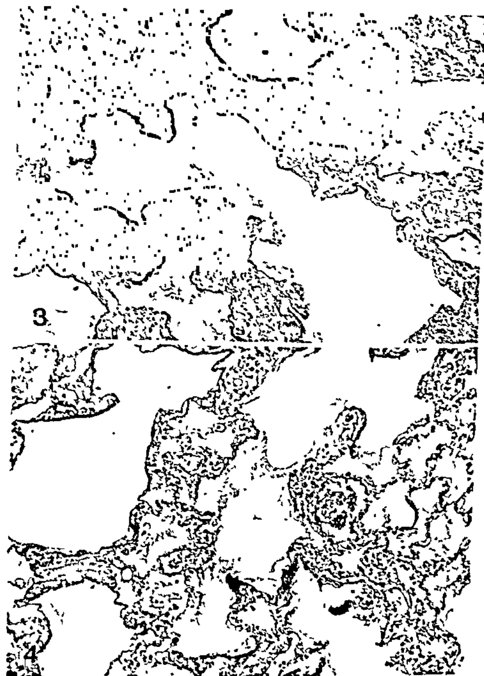
A histological feature common to these cases of hyaline membranes after treatment with oxygen was a frequently large number of unpigmented alveolar phagocytes in and on the septa as well as in the alveoli Sometimes the septa were oedematous and congested Most of the hyaline membranes were fresh but some were obviously in a state of fibrous organization

t

graphic appearances of the lungs In one severe case (640 62) the pulmonary changes were interpreted as pneumonic But no pneumonia was found at autopsy

DISCUSSION

Pulmonary hyaline membranes were first described by *Hocchetm* (1903) In 1932 *Farber et al* drew attention to the occurrence of hyaline membranes in pneumonic lungs in patients with influenza and stressed the significance of mechanical dispersion of exudate in patients with ... in newborns This conclusion was corroborated by observations made in animal experiments with instillation via a tracheal tube of various preparations In a series of investigations of this type other authors have succeeded in producing hyaline membranes in experimental animals (*Laufe et al* 1954 1955 *Mi... urr* 1958) Several authors have claimed that aspiration of amniotic fluid cannot by itself explain all cases of hyaline membranes in newborns (*Hadders* 1955 *Gitlin et al* 1956)



Figs 3–4

Fig 3 (291/63) Widespread hyaline membranes on wall of alveoli Septa oedematous with proliferation of large polymorphous fibroblasts Haematoxylin-eosin × 75

Fig 4 (340/63) Fresh hyaline membranes lining alveoli Some phagocytes in septa Haematoxylin eosin × 120

6 days

S.L. (340/63) An 18 year ... Fallot with left sided anas... obliterated Re-operation ... prosthesis at the site of the septum and excision ... of the pulmonary cone Signs of haemorrhage were observed after the operation and at re operation, some hours later, an opening in the right ventricle due to defective ... The patient became successively worse and died 2 days after

partly oedematous septa and some phagocytes in the alveoli In some areas the alveoli contained eosinophilic oedematous fluid In some areas small bronchopneumonia were seen around the bronchi

Oxygen treatment (in addition to that at operation) about 30 per cent oxygen mixture for 12 hours, 40–53 per cent for about 24 hours, and 70 per cent for 5 hours

A histological feature common to these cases of hyaline membranes after treatment with oxygen was a frequently large number of unpigmented alveolar phagocytes in and on the septa as well as in the alveoli Sometimes the septa were oedematous and congested Most of the hyaline membranes were fresh but some were obviously in a state of fibrous organization

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In electron microscopic studies *van Bremen et al* (1957) found that the hyaline membranes in lung tissue from premature infants and from guinea-pigs treated with oxygen showed a similar histological picture and consisted of fibrinous material They detected local injury of the membrane of the alveolar capillaries, probably sites of transudation Similar injuries have also been demonstrated in lungs of mice by *Cedergren et al* (1959)

Pattle (1955) and *Avery et al* (1959) stressed the importance of adequate surface tension of the thin film of fluid covering the alveolar epithelium The surface tension is thus decreased by a lipoprotein (Clements 1962) which is invariably missing in prematures and in infants with hyaline membranes The absence of this lipoprotein results in an increased surface tension with consequent collapse of the alveoli and capillary leakage

A recently discovered enzymatic defect interfering with normal fibrinolysis in prematures and in infants with hyaline membranes (*Liebermann* 1959, 1961, *Liebermann et al* 1960) and often due to the presence of an inhibitor has attracted much attention The absence of fibrinolysis results in the development of hyaline membranes from alveolar transudate

It is known that treatment with highly concentrated oxygen can induce the formation of hyaline membranes in experimental animals (*Clamann et al* 1940, *Liebegott* 1941, *Pichotka* 1941, *Bruns et al* 1954, *Buckingham et al* 1960) Animals which survived such treatment and were killed some time later did not present any pulmonary changes, which thus argues for the reversibility of the pulmonary injury Increased permeability of the capillaries of the lungs may result in effusion and precipitation of fibrin with the formation of hyaline membranes as a result (*Ariwaka* 1956, *Berfenstam et al* 1958) The impairment of the permeability may be due to a toxic effect of the oxygen

Pulmonary changes have also been described after oxygen treatment of adults In a small autopsy series *Pratt* (1958) found capillary stasis and proliferation after treatment for 2 days with oxygen and diffuse fibrosis after treatment for about 2 weeks The pulmonary changes were regarded as probably being reversible at least before development of fibrosis No hyaline membranes was seen in his series in which oxygen had been given only by nasal catheter

The fibrinolytic factors have also been studied in adult autopsy series (*Capers et al* 1964) The findings suggested a mechanism similar to that described above for newborns The possibility of an abnormally high surface tension in adults with pulmonary hyaline membranes has also been discussed The validity of the fibrinolysis theory has recently been questioned (*Sandberg et al* 1964) In a large autopsy series comprising 1260 subjects *Capers* (1961) found hyaline membranes in 37 (2 76 per cent) Among these, 13 had received oxygen treatment to which, however, no aetiological importance was attached

Radiation treatment produces a characteristic histological picture of the lung tissue (*Warren et al* 1940), of which hyaline membranes are a component Both oxygen treatment *and radiation* treatment induce a release of oxidizing free radicals (*Gerschman et al* 1954) which interfere with enzyme systems and nucleic acid metabolism *(Buckingham et al* 1960)

The findings in the present material suggest a causal relationship between treatment with oxygen and the development of hyaline membranes Thus, among 131 patients treated with oxygen (lowest concentration 40 per cent) hyaline membranes were found in as many as 11 But the pulmonary changes were widespread only in 3 of these 11 cases In one of these cases the oxygen content of the gases was at most 40 per cent but treatment was fairly long, all together 13 days, and in the remaining 2 cases the subjects had *received oxygen in a concentration* of at least 50 per cent for 2 days in one and for 5 days in the other case

but the fact

n concentra-

ygen is given

via a nasal catheter the actual concentration of the oxygen in the gases inspired can only be roughly estimated With such simple arrangement the concentration of the oxygen in the inspired gases will exceed 50 per cent only in rare cases Our observations suggest that continuous administration of oxygen in a concentration exceeding 50 per cent or administration of oxygen in a concentration of 40 per cent for a long time *involves the risk of causing pulmonary hyaline membranes*

SUMMARY

One hundred and fifty subjects from a respiratory centre were examined post mortem for the occurrence of pulmonary hyaline membranes Among these 131 had received oxygen treatment, usually in a respirator for a varying length of time 14 out of the 28 subjects in whom hyaline membranes were found had had pulmonary infections, uraemia or radiation reactions, which might explain the hyaline membranes Among the remaining 14 cases 3 patients with extensive pulmonary changes are described *in detail These observations suggest* that prolonged treatment with oxygen in high concentration can be of significance in the causation of hyaline membranes

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CYSTIC DISEASE OF THE KIDNEYS

Autopsy Report and Family Study

By

O Heggo and J B Natvig

Received 8 ii 65

A specific type of cystic disease of the kidneys is the so-called Potter s hamartomatous variety (*Potter* 1961) *Heggo & Natvig* (1963, 1965) presented a microdissection study of this variety and concluded that the primary disturbances affected the interstitial part of the collecting system producing a hyperplastic proliferation of epithelium with cyst formation Moreover in accordance with Potter's experience, there was hyperplasia of the epithelium in the bile-ducts

The condition is scarcely compatible with life of any duration and affected infants die within few hours after birth

It has been suggested that hereditary factors are present in this and other types of cystic kidneys and many attempts to elucidate this question are reported in the literature (reviewed by *Dalgaard* 1957) However the pathological descriptions and classifications are inadequate in many of the genetic studies

The object of the present work is to confront the autopsy findings in the cases discussed in the microdissection study with the known structural changes and to give a family study

MATERIAL AND METHODS

Autopsy was performed at the Gade Institute on five infants within 24 hours of death The organs including the brain and cerebral blood vessels were examined macroscopically The parenchymatous organs were fixed in formalin and microscopy was performed on sections stained with haematoxylin and eosin

The cases originated in 3 families in which 151 persons in 4 generations were examined anamnestically for stillbirths neonatal deaths malformations renal diseases and consanguinity The urine was analysed (protein pus sugar blood spec g pH) in parents and siblings of the autopsy cases in two of the families

RESULTS

The mothers of the five autopsy cases were healthy they were rhesus positive had negative Wassermann reactions and had no embryopathic diseases during the pregnancy The mother of case III took 20 tablets of Postafen (meclozini chloride 0 025 g and was treated with sulphonamides for acute pyelonephritis during the fourth month The deliveries were uncomplicated and the newborn children breathed

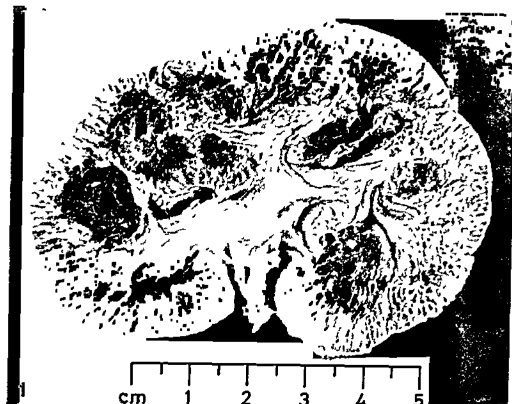


Fig 1

Right kidney case III Cross section showing the typical spongy appearance of the enlarged kidney Cortex and medulla can be distinguished No malformation of the pelvis

well for a short period Within periods lasting for some minutes up to a couple of hours, they developed asphyxia and died within 2 up to 31 hours after birth, despite clearing of the air passages and oxygen therapy Bilateral abdominal enlargement and pulmonary atelectasis were found clinically One of the infants was seen to urinate (case III) Urograms were not taken and blood urea or creatinine not determined in the autopsy cases

Post mortem Examination

The body weights and lengths, and some organ weights are shown in Table 1 None of the bodies showed external malformations

1) with smooth external surfaces and stripped The cortex seemed to be of

(*Fig 2*), *giving the kidneys a characteristic spongy appearance* enlarged corresponding to the kidney enlargement but the calyces and papillae appeared normal in shape On each papilla 10 to 18 openings were seen some of which were abnormally wide No obvious abnormality was found in the ureters or bladders In case IV the bladder contained some urin like fluid

Microscopic examination of the kidneys revealed that the cysts were lined by a cuboidal cells (Figs 3 4 6) The cells were sharply outlined with

Y-shaped division towards the external

TABLE 1

Autopsy Cases Body Weights and Lengths Weights of Lungs Livers and Kidneys, Sex Date of Birth and Postnatal Life *

Case no Sex / Date of birth	I Female / Dec 195[illegible]	II Male / June 1958	III Female / March 1962	IV Female / April 1962	V Female / Oct 1962
Postnatal life	2 hrs	31 hrs	2½ hrs	2½ hrs	9 hrs
Body weight	3660 g	2380 g	3400 g	3310 g	2660 g
Length	51 cm	44 cm	49 cm	48 cm	46 cm
Both lungs	42 g (58.0)	29 g (43.6)	26 g (54.9)	40 g (54.9)	33 g (48.9)
Liver	180 g (178.1)	70 g (98.1)	190 g (155.1)	165 g (155.1)	115 g (127.4)
Both kidneys	250 g (28.5)	175 g (19.9)	210 g (25.3)	260 g (25.3)	140 g (23.0)

* The values in parentheses are normal values according to body weight (*Potter* 1961)

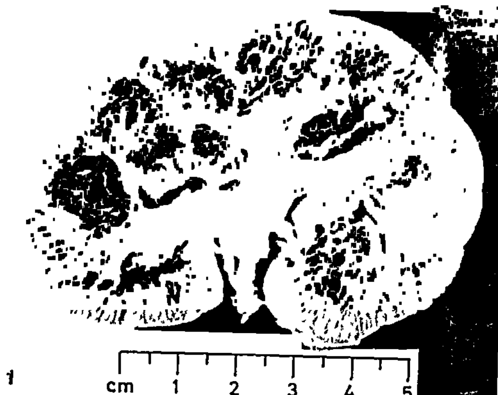


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[illegible]

Y shaped division towards the external su[illegible]



Fig 5

kidney case IV Well developed glomerulus connection to the proximal convoluted tubule at the lower left side Upper left side Part of the nephron with regular cylindrical epithelium (Haematoxylin and eosin × 560)



Fig 6

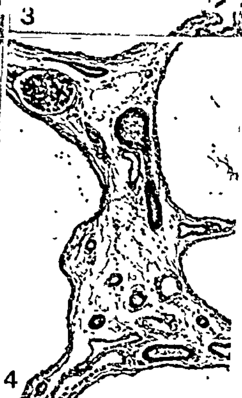
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× 560)

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Figs 2–4

Fig 2 Kidney case IV Cysts are seen from just beneath the capsule to the papilla Glomeruli are scattered in the intercystic island Two papillary ducts open into the pelvis (Haematoxylin and eosin × 14)

Fig 3 Kidney case II Relatively few cysts and fairly well preserved structure (Haematoxylin and eosin, × 140)

Fig 4 Kidney case IV Juxtamedullary glomerulus parts of the nephron with relatively dark cubocylindrical epithelium The narrow channels with epithelium very similar to that of the cysts are part of the collecting system (Haematoxylin and eosin × 140)



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K dn y a e IV Well de eloped glomerulu connect on to the prox mal con oluted ubule at the low r left s de Upper left s de Part of the nephron th regular yl nd cal ep hel um (Haematoxyl n and eos n × 560



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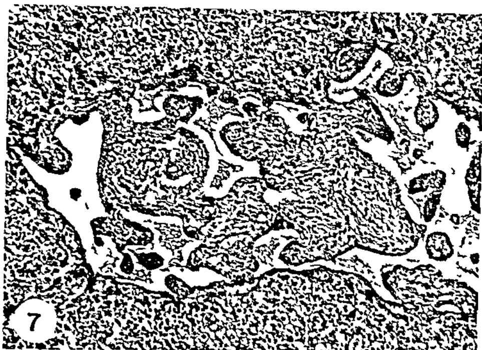


Fig 7

Liver case IV Marked cystic hyperplasia of the small bile ducts The papillary projections are filled with connective tissue (Haematoxylin and eosin × 140)

centration of connective tissue around the cysts The renal corpuscles showed no dilatation of the Bowman's capsules and the glomeruli had a well developed capillary *network Tubules with* a cuboidal acidophilic epithelium appeared to open into capsular spaces (Fig 5) Similar tubules, with a slightly lower epithelium were in some instances connected with the cystic cavities described (Fig 6) Furthermore some tubules were identified as loops of Henle On the whole, these portions of nephrons showed no obvious signs of cystic formation In addition normal or slightly dilated collecting tubules with an epithelium similar to that of most of the cysts were seen from cortex to papillae

The lungs were atelectatic with firm surfaces In cases II and V the hyaline membranes had developed

The livers were of normal size and shape The small bile ducts were dilated and tortuous, and the epithelium hyperplastic with papillary projections into the lumina (Fig 7) Periportally the connective tissue was increased, corresponding to the abundance of epithelial ducts

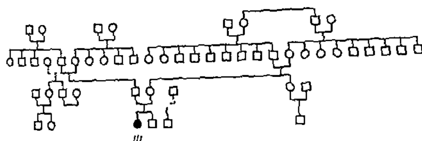
The remaining organs including the brain and cerebral blood vessels appeared normal Urin from cases III and IV was unfortunately not collected for examinations

FAMILY STUDY

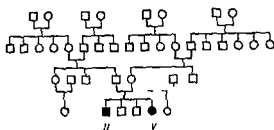
The family trees are shown in Fig 8 Apart from the affected sibships, cases of neonatal death, cystic kidneys or deaths attributed to renal or cardiovascular diseases were not found among children and young adults Except for family A, where two of the grandparents were first cousins, no consanguinity was known However, all the parents of the affected sibships traced their ancestors back to the same district in Western Norway (Sogn and Fjordane)

■ ● Affected
□ ○ Unaffected
⊗ Abortion
⊘ Still born

Family A



Family B



Family C

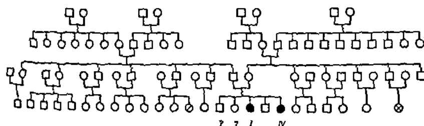


Fig 8

Genealogy of the autopsy cases. Squares and circles indicate males and females respectively. The two cases with questionmark died of unknown causes probably prematurity (see text)

Family A.

Mother, born in 1934 Urine physiological Blood urea and creatinine normal In 1954 she had one boy whose father was not the same as the father of the affected child In 1963 she gave birth to a boy in whom evidence of kidney or liver affection was not observed The father and both boys were clinically healthy and had physiological urine

Family B

Mother, born in 1939 Urine physiological Pyelograms negative She had two boys in 1959 and 1960, respectively, both being clinically healthy with physiological urine In 1964 she was delivered of a girl who had another father than the affected children The infant was clinically unaffected, with physiological urine and normal urograms The fathers were clinically healthy

Family C

Mother born in 1923 Urine physiological Her two first children, born in January and in December 1951, were born at home about 4 weeks before term and died after ½ and 1 hour, respectively, but no further information was available She gave birth to a boy in 1960 The father and this boy were clinically unaffected and healthy In the mother's family one abortion had occurred, but further information about this case was not available In the father's family there was one stillborn, without evidence of malformations

DISCUSSION

The gross picture of the bilaterally enlarged kidneys was very uniform with innumerable small cysts arranged characteristically, giving a spongy appearance unlike other types of cystic kidneys It thus presents a valuable aid in identifying the variety Close inspection gave the impression that the majority of cysts were derived from the collecting system A further important finding was that the pelves, as well as the calyces and papillae, were well formed

Microscopic investigation seemed to confirm that the cystic cavities belonged to the collecting system Thus, tubules appearing to be distal convolutions of normal nephrons could be seen to open *into* cysts, and in addition there were cavities that opened into the pelvis The cells lining the majority of the cysts were high cuboidal and did not give the impression of increased pressure within the cysts The renal corpuscles appeared normal as did the nephrons as a whole There was no increase of connective tissue around the cysts or in the kidneys generally

In all cases, a hyperplastic proliferation of bile duct epithelium with cyst formation was seen

All these findings indicated that we were dealing with Potter's hamartomatous variety of cystic kidneys

The cause of death was uncertain However, life was too short for the development of uraemia *Norris & Herman* (1941) pointed to the large kidneys as the cause of impaired inspiration, pulmonary atelectasis and death This may at least partly explain death in the present cases In addition there might have been important changes in electrolytes and body fluids, but these were not investigated Although there was connection between nephrons, cysts and pelves (*Heggo & Natvig* 1963, 1965), the extended structural alterations of the majority of nephrons, probably must have influenced tubular filtration and reabsorption The liver changes, although widespread, were scarcely profound enough to explain the early deaths

From the family study, a hereditary factor was strongly suggested in the type of cystic kidneys presented Besides the three propositi (cases III, IV, and V) only two similar cases could be found in the autopsy material from 1950 to 1964 in The Gade Institute That these cases (cases I and II) turned out to be siblings of two of the propositi was not likely to be pure chance Embryopathic diseases, chemicals, and irradiation could be excluded with high probability It was unlikely that treatment with sulphonamides and Postafen of the mother in family A would have caused the cystic changes in her infant

In the investigations of *Bunting* (1906), *Berner* (1913), and *Marquard* (1934) were siblings in which the changes described seem to be in accordance with the hamartomatous variety

Potter (1961) stated that among the neonatally observed different types of cystic kidneys the hamartomatous variety was inherited and, in her experience the only one found in siblings

Ferguson (1949) in his comprehensive study found no affection neither clinically nor in pyelograms, of any of the parents of the infantile cases (8 families) Affected parents (adult cases) did not give birth to neonatal cases, but families in which neonatal cases already had occurred were prone to further affliction However, the description of the structural changes in the infantile cases does not give a clear understanding of the type or types investigated

Many authors suggest, as *Dalgaard* (1957), that this variety of cystic kidneys is recessively inherited However, his cases with large ' spongy" kidneys might have included not only the hamartomatous variety, but also other types

The suggestions in the literature that the type of cystic kidneys here presented is recessively inherited are based mainly on the occurrence in siblings whose parents were unaffected However, the more detailed genetic studies lack adequate morphological description

The five cases in the present study is a rather small number for statistical support in the determination of the type of heredity, espe-

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In all cases, a hyperplastic proliferation of bile duct epithelium with cyst formation was seen

All these findings indicated that we were dealing with Potter's hamartomatous variety of cystic kidneys

The cause of death was uncertain However, life was too short for the development of uraemia *Norris & Herman* (1941) pointed to the large kidneys as the cause of impaired inspiration, pulmonary atelectasis and death This may at least partly explain death in the present cases In addition there might have been important changes in electrolytes and body fluids, but these were not investigated Although there was connection between nephrons, cysts and pelves (*Heggo & Natvig* 1963, 1965), the extended structural alterations of the majority of nephrons, probably must have influenced tubular filtration and reabsorption The liver changes, although widespread, were scarcely profound enough to explain the early deaths

From the family study, a hereditary factor was strongly suggested in the type of cystic kidneys presented Besides the three propositi (cases III IV, and V) only two similar cases could be found in the autopsy material from 1950 to 1964 in The Gade Institute That these cases (cases I and II) turned out to be siblings of two of the propositi was not likely to be pure chance Embryopathic diseases, chemicals, and irradiation could be excluded with high probability It was unlikely that treatment with sulphonamides and Postafen of the mother in family A would have caused the cystic changes in her infant

In the investigations of *Bunting* (1906), *Berner* (1913) and *Marquard* (1934) were siblings in which the changes described seem to be in accordance with the hamartomatous variety

Potter (1961) stated that among the neonatally observed different types of cystic kidneys the hamartomatous variety was inherited and, in her experience the only one found in siblings

Ferguson (1949), in his comprehensive study found no affection neither clinically nor in pyelograms of any of the parents of the infantile cases (8 families) Affected parents (adult cases) did not give birth to neonatal cases but families in which neonatal cases already had occurred were prone to further affliction However the description of the structural changes in the infantile cases does not give a clear understanding of the type or types investigated

Many authors suggest as *Dalgaard* (1957), that this variety of cystic kidneys is recessively inherited However, his cases with large "spongy" kidneys might have included not only the hamartomatous variety, but also other types

The suggestions in the literature that the type of cystic kidneys here presented is recessively inherited are based mainly on the occurrence in siblings whose parents were unaffected However the more detailed genetic studies lack adequate morphological description

The five cases in the present study is a rather small number for statistical support in the determination of the type of heredity, espe-

Family A

Mother, born in 1934 Urine physiological Blood urea and creatinine normal In 1954 she had one boy whose father was not the same as the father of the affected child In 1963 she gave birth to a boy in whom evidence of kidney or liver affection was not observed The father and both boys were clinically healthy and had physiological urine

Family B

Mother born in 1939 Urine physiological Pyelograms negative She had two boys in 1959 and 1960, respectively, both being clinically healthy with physiological urine In 1964 she was delivered of a girl who had another father than the affected children The infant was clinically unaffected, with physiological urine and normal urograms The fathers were clinically healthy

Family C

Mother, born in 1923 Urine physiological Her two first children, born in January and in December 1951, were born at home about 4 weeks before term and died after ½ and 1 hour, respectively, but no further information was available She gave birth to a boy in 1960 The father and this boy were clinically unaffected and healthy In the mother's family one abortion had occurred, but further information about this case was not available In the father's family there was one stillborn, without evidence of malformations

DISCUSSION

The gross picture of the bilaterally enlarged kidneys was very uniform with innumerable small cysts arranged characteristically, giving a spongy appearance unlike other types of cystic kidneys It thus presents a valuable aid in identifying the variety Close inspection gave the impression that the majority of cysts were derived from the collecting system A further important finding was that the pelves, as well as the calyces and papillae, were well-formed

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In all cases, a hyperplastic proliferation of bile duct epithelium with cyst formation was seen

Multifactoral inheritance is most often found in characters with a broad spectrum of variance. The uniformity of affection in the present cases makes this form very unlikely.

This investigation thus gives much support to the statement that Potter's hamartomatous variety of cystic kidneys is due to simple *single* factor recessive inheritance.

Although no hereditary connection has been established between adult and infantile cases of genetically determined cystic kidneys, it is still possible that the genes in question are allelomorphic (due to different mutations in the same gene locus).

Analysis of genetic markers was scarce and material for chromosome analysis was not taken.

SUMMARY AND CONCLUSIONS

The autopsy findings of five cases of Potter's so-called hamartomatous variety of cystic kidney disease are presented. Based on the authors' microdissection study, which has shown that the cysts are derived from the collecting system, the gross and microscopic features of *importance for a correct pathological diagnosis are stressed.*

The family studies sustain the suggestion of simple single factor recessive inheritance in this variety.

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Dominant inheritance might be explained by the existence of very low penetrance, or occurrence of subclinical cases, in at least three generations in each of the three families This, however, is unlikely, according to the early onset and severe affection of the verified cases

Among the autopsy cases were four girls and one boy, the four living siblings are all boys Whether the sex skewness is real or a mere coincidence can be determined only by further investigation However, recessive sex linkage inheritance can be excluded from these ratios

The occurrence of two pairs of affected siblings whose parents and other relatives in four generations were clinically normal and healthy, indicate recessive inheritance No consanguinity between the parents of the affected children has been found All the parents, however, could trace their families back to a certain area in Sogn and Fjordane This may add some proof to the possibility of consanguinity Two of the mothers had clinically unaffected children by other husbands The finding of these children unaffected might, however, have happened also in the case of dominance

The proportion of verified affected cases to all children in the three sibships is five to eleven As pointed out clearly by *Stern* (1949), great deviations from the expected "classical ratios" will be encountered especially when recessive traits are involved because we are unable to observe families in which the heterozygote parents have no affected children The corrected expectation is $q^1 = \frac{q}{1 - p^n}$ (q is *a priori* the expected number of homozygote recessive children (1/4), p that for homo- and heterozygote dominant children (3/4) and n the number of siblings) Using the correction, the expected number of affected children in family A is 1 146, in family B 1 463, and in family C 1 640 This gives 4 25 *expected children against 5 observed*

Chi-square after correction (see *Stern* (1949)) for expected values below five is estimated $\chi^2 = 0\ 03$ On the hypothesis of simple single factor, this gives more than 95 per cent probability that the observed value indicates a recessive inheritance However, this presupposes complete information of each sibship Two siblings in family A were uncertain If both had the same affection as the other cases the χ^2 would be 2 6, which gives a probability of slightly more than 10 per cent that this value according to recessive inheritance should have been reached by chance As the two children died only ½ and 1 hour after birth and both were born four weeks before term, it is possible that the causes of death were complications of prematurity Among the affected children the most premature one lived longest and had the mildest kidney changes, but general tendencies cannot be drawn *from these* few cases

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. . . with a surgical clamp Four other

. . . chloroacetic acid . . .

TABLE 1

Antibody Response of Bursectomized and Unbursectomized Animals

Assay	Unbursectomized	Bursectomized	Probability
Antigen Clearance (Days)	4 3 ± 0 08* (30)§	4 9 ± 0 65 (24)§	P < 0 001
Induction Period (Days)	5 5 ± 0 01 (24/30)†	6 4 ± 0 29 (5/24)†	P < 0 005

The radioactivity was measured in a liquid scintillation counter (Packard Tri-Carb 314 ex) at a temperature of +2° C utilizing Bray's solution as scintillator (*Bray* 1960) to which 10 per cent water phase (nucleic acids in 5 per cent TCA) was added Nucleic acids isolated in the same way from corresponding organs of non injected animals were used for background correction The channels ratio method (*Baillie* 1960) was used for quenching correction All samples were used in duplicates and the duplicates were counted twice In the determinations of specific activity (spec act) the average of the four values of each sample was used The specific activity is expressed as counts/min µg DNA

Experiment II *Local Labelling and Bursectomy*

Forty five two weeks old white leghorns were intrabursally injected with 25 µC of H³ thymidine in a volume of 0 05 ml as described above Four hours after injection twenty one of the animals were chosen at random and bursectomized Sham operations were performed on the remaining twenty four animals At two, four and six days after intrabursal injection eight of the unbursectomized seven of the bursectomized and one uninjected control animal were chosen at random and sacrificed The thymus spleen liver a one inch strip of the large intestine, beginning one inch from the cloaca and extending anteriorly and the bursa when present were collected and processed in the manner described above The specific activities of each organ taken from the bursectomized groups at each sacrifice period were compared with those of the corresponding unbursectomized groups The data were statistically analysed using the Fischer Student t test

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THE TRANSPORT OF CELLS FROM THE BURSA OF FABRICIUS TO THE SPLEEN AND THE THYMUS

By

ROY WOODS[1] and JUHANI LINNA

Received 4 ii 65

The immunological effect of the extirpation of the bursa of Fabricius from young chickens has been studied by numerous investigators (*Glick et al* 1956, 1961, *Chang et al* 1957, *Warner et al* 1962, *Mueller et al* 1960) All of these studies have indicated that such bursectomized animals are "immunological cripples"

In many respects, the action of the bursa parallels that of the thymus in mammals Consequently, speculations concerning the specific roles of these two lymphoid structures have been similar

These investigations were designed in order to 1) determine if the cells of the bursa of Fabricius could be locally labelled with tritiated thymidine, and if so, 2) to study the possible transport of cells from the bursa to other lymphoidal organs

MATERIALS AND METHODS

To be sure that the bursa of Fabricius is of the same immunological importance in the strain of chickens used in our laboratory 24 white leghorns were bursectomized at 1 week of age following the procedure of Mueller (*Mueller et al* 1960) Five weeks later all animals were BSA) per kg body weight All animals The sera were tested by the capillary flocculation test (*Woods 1963*) in order to determine the presence of circulating antigen and antibody A high titered chicken anti BSA was used to test for the presence of circulating antigen and a 0 06 per cent solution of BSA was used to test for the presence of antibody The results (cf Table 1) show that bursectomy in our hands has the established effect on precipitin production in the strain of chickens which is used in our laboratory

Experiment I *Local Labelling of Bursa with Tritiated (H^3) Thymidine*

Five six week old white leghorns were anesthetized with ether and the bursa exposed in the same manner as when bursectomy is performed With a No 26 needle 0 05 ml of H^3 thymidine (2 9 C/mM specific activity) was carefully injected into the parenchyma of the organ Each animal received 25 μC of the isotope Care was taken not to penetrate into the central area of the bursa After gentle wiping of the bursa

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[1] United States Public Health Service Postdoctoral Research Fellow Fellowship Number 1 F2 AI 20 340 01 On leave from Miles College Birmingham Alabama USA

The work has been supported by the *Swedish Medical Research Council*

two days after injection In no case was there a *statistically significant* difference between the specific activities of the corresponding organs when comparisons were made between the bursectomized and unbursectomized animals As shown in Table 4, the results for four days after injection also revealed no significant difference between the two groups of animals Table 5 records the results obtained on the sixth day after injection It can be seen that the specific activities of the spleen and thymus in the unbursectomized animals were significantly greater than those shown for the corresponding organs in the bursectomized group On the other hand, the specific activities of the liver and intestine did not differ when the two groups were compared

TABLE 4

Specific Activities of Various Organs after Intrabursal Injection of H^3 Thymidine (Four Days after Injection)

Organ	Specific activity ± standard error	Probability
Bursa	152 ± 9 1	
Liver (B)	16 ± 5 0	P < 0 2
Liver N	9 ± 1 9	
Intestine (B)	20 ± 5 7	P < 0 5
Intestine N	15 ± 4 0	
Thymus (B)	7 ± 1 4	
Thymus N	7 ± 2 0	
Spleen (B)	16 ± 6 1	
Spleen N	17 ± 6 6	P > 0 5

Abbreviations see Table 3

TABLE 5

Specific Activities of Various Organs after Intrabursal Injection of H^3 Thymidine *(Six Days after Injection)*

Organ a	Specific activity + standard error	Probability
Bursa 2nd Day*	207 ± 70	
Bursa 6th Day	40 ± 16	P < 0 05
Liver (B)	10 ± 1 8	
Liver N	13 ± 3 8	P < 0 5
Intestine (B	12 ± 1 4	
Intestine N	16 ± 4 1	P < 0 4
Thymus (B)	4 ± 0 92	
Thymus N	8 ± 1 69	P < 0 025
Spleen (B)	13 ± 1 69	
Spleen N	23 ± 3 3	P < 0 025

* Refers to the day after intrabursal injection
Abbreviations see Table 3

DISCUSSION

Inasmuch as the bursa does not appear to produce antibody (Thorbecke et al 1957), it might supply cells to other organs which possess

RESULTS

Experiment I *Local Labelling of Bursa with H³-Thymidine*

Table 2 records the specific activities of the various organs examined It can be seen that the specific activity of the bursa after intrabursal injection was approximately twenty times greater than after intravenous injection Also, it can be seen that the specific activities of the spleen, thymus and intestine were significantly higher after intravenous than after intrabursal injection After intrabursal injection the specific activities of the spleen, thymus and intestine were respectively 2.6 per cent, 0.81 per cent and 2.4 per cent of the specific activity of the bursa

TABLE 2

Organ	Specific activity ± standard error	Probability
Bursa IB	258 ± 45.0	
Bursa IV	13.5 ± 0.67	
Spleen – IB	6.73 ± 1.0	$P < 0.005$
Spleen – IV	14.93 ± 1.6	
Thymus – IB	2.1 ± 0.34	$P < 0.001$
Thymus IV	4.23 ± 0.17	
Intestine – IB	6.18 ± 1.0	$P < 0.001$
Intestine – IV	17.49 ± 2.5	

IB = intrabursal injection IV = intravenous injection

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Specific Activities of Various Organs after Intrabursal Injection of H³ Thymidine (Two Days after Injection)

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Bursa	207 ± 70	
Liver – (B)*	16 ± 2.9	$P > 0.5$
Liver N§	31 ± 15.0	
Intestine (B)	32 ± 12.4	
Intestine N	32 ± 8.3	
Thymus (B)	13 ± 5.5	
Thymus N	13 ± 4.9	
Spleen (B)	27 ± 8.7	$P > 0.5$
Spleen N	55 ± 24.0	

* (B) = The animals were bursectomized approximately four hours after intrabursal injection

§ N = These animals were injected intrabursally but were not bursectomized

Experiment II *Local Labelling and Bursectomy*

These are the results of the comparisons of the specific activities of the spleen, thymus, intestine and liver between the bursectomized and unbursectomized groups of animals on days 2, 4 and 6 after intrabursal injection of 25 μC of H³-thymidine Table 3 records the results obtained

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Abbreviations see Table 3

DISCUSSION

Inasmuch as the bursa does not appear to produce antibody (*Thorbecke et al 1957*), it might supply cells to other organs which posses

the environment appropriate for the differentiation of immunologically competent cells

Experiment I represented an attempt to develop a model which would allow for a study of the possible transport of cells from the bursa If it were possible to locally label DNA in the cells of the bursa of Fabricius, a technique for the study of cell migration would be created The results of experiment I indicate that it is possible to obtain a high degree of labelling of bursal cells with H^3 thymidine by carefully injecting the isotope into the parenchyma of the organ Even though one obtains a high degree of local labelling by this method, a small amount of leakage of the isotope from the injected bursa is inevitable In order to adequately study the transport of labelled cells from the bursa, an experimental model was needed, which would provide a way around this apparent difficulty The model used in experiment II was based on the fact that the H^3-thymidine which might leak from the bursa would be incorporated into cellular DNA in secondary organs within four hours (*Cronkite et al* 1959, *Rubini et al* 1960) after injection Half of the animals could then be bursectomized and comparisons could be made between the specific activities of various organs taken from bursectomized and non bursectomized animals at appropriate periods The results obtained with this model indicated that, among the intervals during which determinations were made, only on the sixth day after intrabursal injections could differences be noted between the specific activities of the spleen and the thymus taken from the two groups of animals The specific activities of the spleen and thymus were significantly higher in the unbursectomized groups of animals

Experiment II was intended to demonstrate the possible transport of cells from the bursa of Fabricius to other organs What has been demonstrated, however, is that label has been brought from the bursa to the spleen and the thymus This label could have been brought there by cells or by DNA break down products However, the reutilization of DNA break-down products from all organs should be expected to be high in the intestine (*Bryant* 1964) Thus, a lower specific activity in the intestine of the bursectomized animals than in the non bursectomized ones should have been expected, if there had been a transport of DNA break-down products from the bursa This was not the case, and thus we consider the most possible explanation to be that there is a transport of cells from the bursa of Fabricius to the thymus and the spleen during the experimental period—the cells are "homing" in these organs Our experiments give no information relative to the ultimate fate of these immigrating cells There are two main possibilities to be considered the cells can become a part of the cellular population, or the labelled DNA can be reutilized in these organs

As far as we know, a transport of cells from the bursa to the thymus and the spleen has not been shown previously A similar mechanism of cell transport from the thymus to the spleen (*Fichtelius & Diderholm*

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Our experiments do not exclude the existence of a humoral factor of bursal origin which may be involved in the immune response The technique of local labelling of the bursa with an isotope provides a model for the further elucidation of the fate of bursal lymphocytes

SUMMARY

A technique of local labelling of bursal cells with tritiated thymidine has been developed Utilizing a combination of local labelling of the bursa of Fabricius and bursectomy, it has been shown that there is a transport of cells from the bursa to the spleen and the thymus The immunological importance of this mechanism has been discussed

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DIFFUSE METASTASES IN BRONCHIAL CANCER

By

OVE DENSERT and JAN SÖDERSTRÖM

Received 18 ii 65

have been described in many different kinds of tumours [illegible] appear to be most common in patients with mammary cancer (2, 5, 12, 13, 19, 25), malignant melanomas (3, 9) and small-celled bronchial cancer (2, 7, 22)

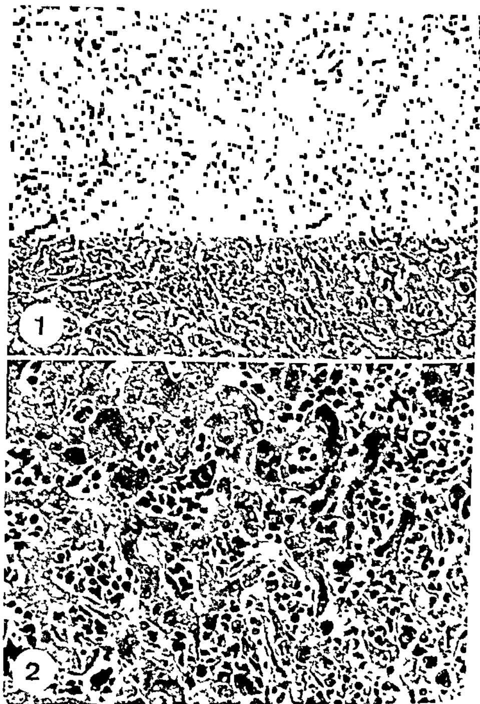
MATERIAL AND METHODS

The material consisted of all subjects necropsied during a 5 year period (June 1 1957 to May 31, 1962) at the Department of Pathology, Malmö Of the 5 711 cases examined malignant tumours were found in 2 165 including 192 with bronchiogenic cancer The material did not include so-called bronchial adenomas or bronchial carcinoids

TABLE 1
Type and Sex Distribution of 192 Cases of Bronchial Cancer

Type of bronchial cancer	Total		♂		♀	
	No	%	No	%	No	%
Squamous cell	73	38	65	44 5	8	17 4
Small-celled	57	29 7	47	32 2	10	21 7
Adenocarcinoma	50	26 1	25	17 1	25	54 3
Undifferentiated	11	5 7	9	6 2	2	4 4
Adenoacanthoma	1	0 5			1	2 2
Total	192	100	146	100	46	100

[illegible] said to be present only when the cancer had infiltrated the entire organ and all histologic sections were of uniform appearance One exception however was case 5 with a finely nodular liver cirrhosis and microscopical



Figs 1 3

Liver Large tumour foci as well as numerous discrete and clustered intrasinusoidal cancer cells giving the preparation an appearance as if it were moulded in a cast *Fig 3* Metastasis in cirrhotic liver

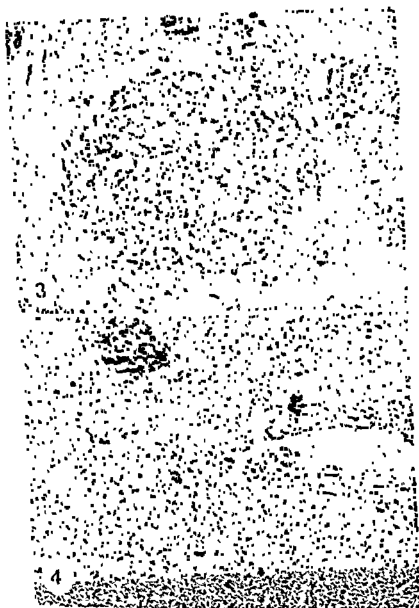


Fig 4

Spleen Numerous discrete and clustered intrasinusoidal cancer cells giving prepara- tion the appearance of a cast Infiltration of vessel walls reminiscent of what is seen in leucosis

ly hepatic cell islets, of which only some were diffusely infiltrated with cancer This case was included because the parts affected by the tumour were of the same microscopic appearance as in the other cases with diffuse metastases and because the incompleteness of the picture of diffuse infiltration probably was due to the cirrhosis

All the clinical and laboratory data were analysed in the cases with diffuse metastases

RESULTS

The incidence and types of metastases in the liver and spleen in the different kinds of bronchial cancer are given in Table 2, from which it is clear that liver metastases were seen in 38 per cent of all cases In small-celled bronchial cancer the frequency was as high as 70 per cent. Metastases to the spleen were also more common in cases of small-celled bronchial cancer than in any of the other types No distinction was made between gross and microscopic tumour metastases It might be mentioned, however, that *Marymont & Gross* (15) found that as many as one third of the metastases in the spleen could not be detected with the naked eye Diffuse metastatic infiltration occurred only in small celled bronchial cancer to the liver in five cases and to the spleen in three (Figs 1–4)

TABLE 2

Incidence of Metastases to Liver and Spleen in Various Types of Bronchial Cancer

	Liver metastases				Spleen metastases			
	Total		Diffuse		Total		Diffuse	
	No	%	No	%	No	%	No	%
Squamous cell	10	13.5	–	–	6	8.1	–	
Small celled	40	70.2	5	8.8	12	21.1	3	5.3
Adenocarcinoma	19	37.3			2	3.9	–	–
Undifferentiated	4	36.4	–	–		–		
Total	73	38	5	2.6	20	10.4	3	1.6

TABLE 3

Cases with Diffuse Metastases

Case			Liver			Spleen			Spine		
No	Age	Sex	Nodules	Diffuse	Wt g	Nodules	Diffuse	Wt g	Macr	Micr	Diffuse
1	64	♂	+	+	4800		+	150	+	—*	—
2	69	♀	—	+	1480	—	—	300	+	+	+
3	62	♂	+	+	3000	—	+	220	+	+	+
4	68	♂	+	+	1800	–	+	115	+	—*	—
5	59	♂	+	+	3150	—	—	160	+	+	+

*Histological preparations not available



Fig 5
Spine Numerous cancer cells discrete and in clusters between trabeculae and blood forming cells

The metastases to the spleen could not be seen macroscopically In the liver the metastases were of varying size and some could be seen with the naked eye Metastasis to the spine had been seen at necropsy in all five cases but histological preparations were available only from three of them (Nos 2, 3 and 5) and in these microscopic examination revealed diffuse infiltration (Fig 5)

It is clear from Table 3 that the weight of the liver was normal only in two cases (Nos 2 and 4) and increased in the others The weight of the spleen was normal in cases Nos 1 and 4 and increased in case No 3

Analysis of the clinical data showed that the duration of survival as from the onset of subjective symptoms on an average was 2 3 months (range 1 month to 5 months) The patients spent only a short time in hospital during the final stage, when progression was rapid The laboratory data showed no significant anaemia except in one case (No 2), in which the last haemoglobin value noted was 5 3 g/100 ml In three cases (Nos 2 3 and 5) the thrombocytes were counted and all three showed thrombocytopenia with values between 18 000 and 38,000 The serum bilirubin which was measured in three cases (Nos 1, 3 and 5), was found to be moderately increased (7 3, 9 07, and 1 2 mg/100 ml) In case 3 the transaminase activity was increased (GOT 335 U and GPT 280 U and alkaline phosphatase 11 U) The GPT in case 5 was 128 U

DISCUSSION

Our material derived from a well defined district with only one hospital, where the necropsy frequency was 98–99 per cent of all patients dying in hospital We have tried to assess the relative frequencies of the various types of bronchial cancer in this material. Frequencies based on surgical series or mixed surgical and necropsy series must for obvious reasons be biased

The ratio of males to females in our material was 3·1 The corresponding figures found in Norwegian series (4, 11) were 2 3 1 and 3 1. 1 and in the material of *Herman & Crittenden* (9) 5 4: 1 Squamous epithelial cancer, small-celled bronchial cancer, and undifferentiated cancer were predominant among males, while adenocarcinoma was equally common in both sexes The frequency of adenocarcinoma was somewhat lower and that of squamous epithelial cancer somewhat higher than in other series (4, 9, 11, 17, 21), while that of small-celled bronchial cancer was about the same The sex distribution of the different types of cancer varied

Grouped according to *Kreyberg*'s (14) classification of lung cancer, the ratio between Groups I and II in our material was 2 6 1 *Christiansen* (4) had 1 6·1 and *Jacobsen* (11) 1 3 1, which suggests a high frequency of adenocarcinoma In a series from Venice (6) the ratio was 2 8. 1

Diffuse infiltration in bronchial cancer (2, 7, 22), in malignant melanoma (3, 8) and in mammary cancer (2, 5, 12,13, 19, 25) has often been reported But these reports are based on single cases or on series collected from non-specified cancer materials (22) and do not allow any assessment of the frequency of diffuse metastases in bronchial cancer

Diffuse metastases to the liver and/or spleen have been considered fairly rare In our cases of bronchial concer such infiltration was seen only in the small-celled type Diffuse metastases to the liver were found in five cases (9 per cent) and to the spleen in three (5 per cent) In cases in which histological preparations of the spine were available, metastases of the same type were also found This type of diffuse metastatic growth was seen only in organs with blood sinusoids The other organs studied in our five cases showed ordinary nodular metastases The spleen has often been described (5, 7) as firm or amyloidlike In our three cases it was said to be "firm" at necropsy

Once the disease has reached a stage requiring hospitalization of the patient, its progress is rapid (19, 22) As in other series on record (22), thrombocytopenia, increased serum bilirubin, and sometimes increased transaminase activity suggesting involvement of the liver had been noted Bronchial cancer had been histologically verified ante mortem in two cases and suspected in one case In the remaining two cases some other malignant tumour had been suspected

Iversen & Hjort (10) found steroid therapy of mammary cancer to be accompanied by a markedly increased frequency of metastases to the spleen, and one of their cases showed diffuse infiltration *Arrazloa et al* (1) found treatment of various types of tumour with steroids and cytostatics to be followed by diffuse metastases Small doses of steroids had been given in our case 1, and case 5 had been treated with a prednisol preparation (Decadron) the diagnosis being hyperadrenocorticism Thus our series had not received any uniform type of treatment, and factors other than steroid or cytostatic therapy appear to be responible for this type of metastases

SUMMARY

192 cases of primary bronchial cancer seen at necropsy were classified according to type Of 57 cases of small celled bronchial cancer diffuse metastases to the liver were seen in 5 (9 per cent), including 3 (5 per cent) to the spleen In 3 of the 5 cases diffuse metastases to the spine were also noted

These cases had not received any uniform treatment Clinically they showed rapid progression

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EMBOLISM OF CEREBELLAR TISSUE TO THE PULMONARY ARTERIES FOLLOWING HEAD INJURY

By

Svein Thunold and Johs Ro

Received 14 ii 65

Thromboemboli are commonly found in the pulmonary vessels at autopsy Occasionally tissue fragments are also found in these vessels but reports of brain tissue in the pulmonary arteries are rare

We have recently examined a case of severe head injury where cerebellar tissue was found in the pulmonary arteries at autopsy The frequency of severe head damage is increasing and in rare instances brain tissue embolism may have been the immediate cause of death

CASE REPORT

S A H a 14 year old boy w
accident He was in deep co
right femur Respiration w
pulse rate 1 0 min The reflexes we
and showed no reaction to light There was a large haematoma in the right temporo parietal region The skin was intact X ray revealed extensive fractures of the skull and skull base and shadows in the basal parts of the right lung A few minutes after
id artificial re-
blood pressure
tient died with

Autopsy (0 710 64) was performed 9 hours after death Only significant findings are reported

Gross examination revealed comminuted fractures of the right temporal and parietal bones most pronounced in the skull base There was a rupture of the dura haemorrhage and herniation of lacerated brain tissue into a fracture through the sigmoidal sulcus There was a severe laceration of the brain tissue both in the cortex and the white matter of the basal parts of the right temporal lobe and in the right half of the cerebellum Parts of the latter were missing Oedema of the brain was marked and there were multiple punctate haemorrhages in the pons and both cerebral hemispheres Slight subarachnoidal haemorrhage was present in the right fronto-temporal area

Fragments of brain tissue were found in the epipharynx and in the right internal jugular vein The lungs were oedematous with numerous

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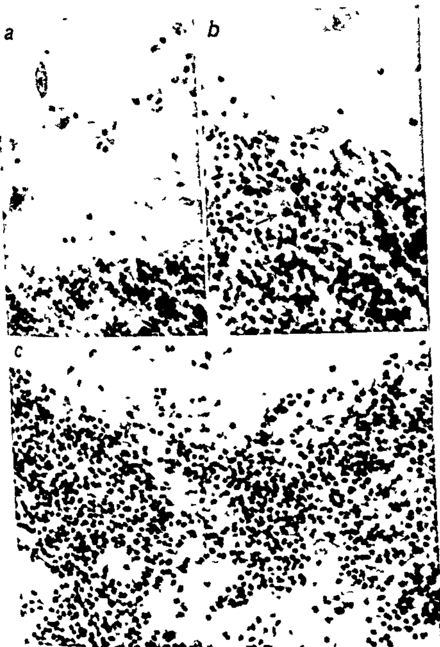


Fig 2

a
b
c

cells

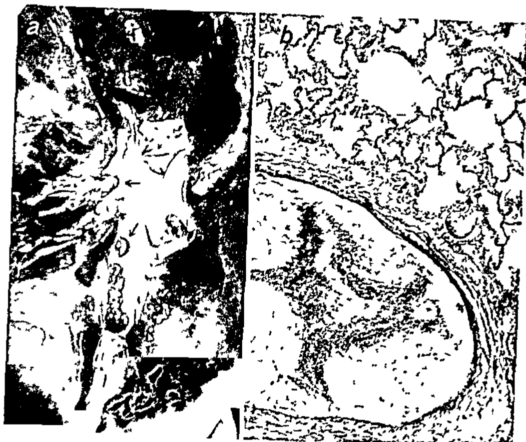


Fig 1

a Part of the left lung with plugs of brain tissue in the pulmonary arteries (→) (gross examination)

b Medium sized pulmonary artery plugged with cerebellar cortex The granular and molecular layer can be seen (Thionin × 60)

subpleural haemorrhagic areas The pulmonary arteries of all sizes contained white plugs resembling brain tissue (Fig 1 a)

Microscopic examination of the brain showed laceration and haemorrhage in the areas mentioned above Only parts of the left dentate nucleus could be found The pulmonary arterial vessels were occluded by fragments of tissue (Fig 1 b) showing the typical histological pattern of the cerebellar cortex The emboli showed the molecular layer with glial cells and neurons both of basket cell and star cell type (Fig 2 a), densely packed granular cells and some large neurons, probably of the Golgi type (Fig 2 b), and some typical though somewhat degenerated Purkinje cells (Fig 2 c) Some emboli contained white matter with numerous glial cells (Figs 3 a) In one of these, big pyramidal shaped nerve cells resembling those of the cerebellar nuclei could be seen (Fig 3 b) All layers contained well preserved capillaries The periphery of some brain tissue fragments contained dense fibrin deposits Small haemorrhagic infarcts with no cellular reaction were found scattered in the pulmonary tissue

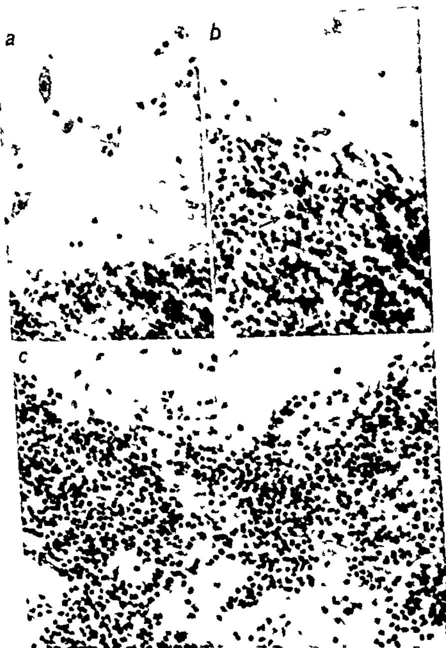


Fig ?

High power magnification (× 3 0 of the embolus seen in Fig 1 b

a. The inner third of the molecular layer with glial cells and some basket cells
b. Densely packed granular cells with one neuron of the Golgi type (→)
c. Three large degenerated Purkinje cells



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revealed brain damage and cerebellar tissue in the left coronary artery In this case foramen ovale was open In addition to traumatic embolism heterotopic brain tissue has been recorded in the lungs of newborn infants with severe cerebral malformations (*Askanazy* 1908 *Hückel* 1929 *Gruenwald* 1941 *Potter & Young* 1942) It has been suggested that such findings are due to brain cells being carried to the lungs by the blood stream either during early foetal life when the brain lesions were developing (*Potter* 1942) or as a result of intrauterine trauma (*Gruenwald* 1941) Destruction of brain tissue and subsequent aspiration to the lungs of amniotic fluid containing brain cells has also been mentioned (*Potter* 1963) It is reasonable to suggest that the brain cells have been carried to the lungs during early foetal life as brain tissue growing in the lungs is not to be expected as a developmental anomaly per se

In only two of the above mentioned cases of severe head injury the brain tissue was visible in the pulmonary arteries on gross examination (*Merkel* 1926 *Oppenheimer* 1954) Direct evidence of the route by which the lacerated brain tissue entered the blood stream was absent in all cases Some authors found ruptures of the dura in the vicinity of the great vessels (*Abrikossoff* 1913 *Merkel* 1926 *Krakower* 1936) but whether brain tissue actually did enter through the defects remained unverified *Tryfus* (1963) found brain tissue in the small veins on the posterior surface of medulla oblongata but could not find any tears in the great veins or sinuses We did not find brain tissue in the ruptured sigmoidal sinus but the finding of brain tissue in the fractured sigmoidal sulcus and the corresponding laceration of cerebellar tissue strongly support the idea that the emboli did enter the blood stream through this sinus

Severe brain injury is frequent as compared to the rarity of reports on brain tissue embolism However among 213 cases of head injury *McMillan* (1956) found 89 with severe brain lesions and in 4 of these brain tissue embolism in the lungs was verified by microscopy In 236 cases of aircraft fatalities *Hickey & Stembridge* (1958) found 3 with cerebral tissue embolism in the lungs Therefore brain tissue embolism following severe head damage may not be so rare as one might expect

SUMMARY

A case of cerebellar tissue embolism to the pulmonary arteries following severe head injury in a 14 year old boy is reported Autopsy findings suggest that the brain tissue entered the blood stream through a ruptured sigmoidal sinus Previously reported cases are briefly reviewed

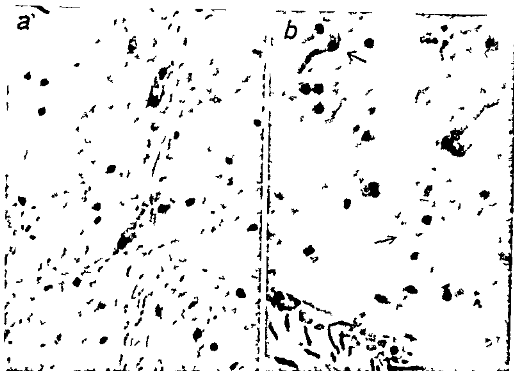


Fig 3

a White matter with glial cells and capillaries
b White matter with large degenerated neurons most probably from one of the cerebellar nuclei (→) Wall of pulmonary artery bottom left (Haematoxylin and eosin × 370)

DISCUSSION

This patient showed ruptures of the dura of the right sigmoidal sinus and lacerated brain tissue was found in the fracture through the sigmoidal sulcus Thus cerebellar tissue from this area has probably entered the circulation through the ruptured sigmoidal sinus The negative pressure in the veins of the head and increased intracerebral pressure probably facilitated this process The histological picture which showed a granular and molecular layer and Purkinje cells was the same as the one normally seen in the cerebellar cortex and thus confirms the cerebellar origin of the emboli Both cortical and white matter were found The haemorrhagic infarcts in the pulmonary tissue show that this process must have taken place a short time before death The brain damage with severe laceration and extensive oedema together with the haemorrhagic infarcts in the lungs are considered to be the cause of death

Only a few cases of brain tissue embolism to the lungs have been reported Most of the reports are concerned with cerebral or cerebellar tissue embolism due to severe head injury (*Merkel* 1926 *Krakower* 1936 *Oppenheimer* 1954 *Nunes* 1955 *McMillan* 1956 *Hickey & Stembridge* 1958 *Tackett* 1964) two reports deal with cerebellar tissue damage and embolism sustained at birth (*Gardiner* 1956 *Tryfus* 1963) *Abrikossoff* (1913) reports a case of a newborn child where autopsy

heterotopic brain tissue has been recorded in the lungs of newborn infants, with severe cerebral malformations (*Askanazy* 1908, *Huckel* 1929, *Gruenwald* 1941, *Potter & Young* 1942) It has been suggested that such findings are due to brain cells being carried to the lungs by the blood stream either during early foetal life when the brain lesions were developing (*Potter* 1942) or as a result of intrauterine trauma (*Gruenwald* 1941) Destruction of brain tissue and subsequent aspiration to the lungs of amniotic fluid containing brain cells has also been mentioned (*Potter* 1963) It is reasonable to suggest that the brain cells have been carried to the lungs during early foetal life as brain tissue growing in the lungs is not to be expected as a developmental anomaly per se

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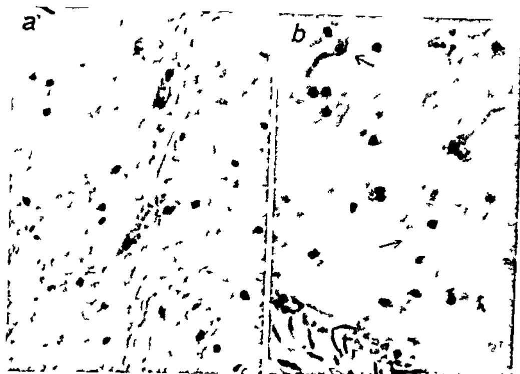


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Department of Pathology I (Professor Jan Mellgren M D),
University of Gothenburg, Sweden

MAST CELLS IN CARCINOID TUMOURS

Distribution, Histochemical Properties and 5-Hydroxytryptamine Content

By

LENNART ENERBÄCK

Received 18 II 65

Currently mast cells are attracting a great deal of attention, primarily owing to the fact that a series of biologically active substances have been demonstrated in their granules It has thus been shown that their metachromatic properties are due to heparin or heparin-like substances (24, 25, 5, 36, 8), it is known too that mast cells from many species contain histamine (34, 10, 32) Moreover, 5-hydroxytryptamine (5-HT, serotonin) is present in mast cells from rats (4) and mice (35) Recently mast cells from hamster, rabbit and cat have also been found capable of taking up and storing catechol amines (1) In addition, mast cells in ruminants have been shown to contain dopamine (20)

The capacity of mast cells to store amines seems to vary greatly in the different spe
tain histamine (
The occurrence

... reptile skin (38) also may contain 5-HT ... (31) and in

Mast cells are widely distributed in the human organism and have been encountered in a variety of tumours by numerous workers

Human carcinoid tumours exhibit a well-documented ability to elaborate and store 5 HT (27, 39) Therefore, since 5-HT forms a histochemically localizable condensation product with formaldehyde, carcinoid tumours would, if they contained a comparatively high proportion of mast cells, offer a favourable environ...
5 HT ... y of any

Acc

mast ... human carcinoids were studied and related to the question ... properties of

of

... indebted to Miss Kerstin Saaborg for skilful technical assistance

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acid disodium phosphate buffer) and at about pH 0 6 (dye dissolved ... Trials showed that suitable staining times were 3 minutes and 3 hours, respectively ... the slides were rinsed in distilled water and then passed rapidly ... xylene and mounted

stained with "Astra"

16 hours (30) Toluidine blue stained sections were destained with acidified ... (10 volumes concentrated HCl to 990 volumes 10 per cent ethyl alcohol)

RESULTS

Enterochromaffin Tumour Cell Reactions

The results of the argentaffin, Schmorl's ferric ferricyanide and the diazo reactions have been reported previously for 23 of the 26 carcinoids in the present series (15) Like these tumours, the 3 additional carcinoids had tumour cells exhibiting both diazo reactivity in the form of brick-red granules and a positive Schmorl reaction manifested by a blue granulation The tumour cells in all the carcinoids displayed a light-yellowish fluorescence, varying in intensity and location within the tumour The tumour cell fluorescence was distinctly visible against the background of the faint light green autofluorescence of the surrounding tissues Compared to the other procedures the fluorescence method gave the impression of having greater sensitivity, since fluorescence could sometimes be detected in areas of some carcinoids where diazo reactivity appeared to be lacking

In the intestinal mucosa investing the carcinoid tumours as well as in the control appendices, the three methods were all capable of visualizing enterochromaffin cells typically situated in the basal layers of the epithelium and showing basal granulation

Mast Cell Distributions in Carcinoid Stroma and in Intestinal Wall

In the stroma of all the carcinoids staining with toluidine blue at pH 4 or pH 0 6 disclosed the presence of cells with metachromatic granules which closely resembled mast cells Similar cells could be demonstrated also in intestinal wall from the carcinoid tissue blocks and in the control appendices At the higher pH level the granulation of these cells appeared dark violet against a clear blue basophilia of cell nuclei in connective tissue and tumour tissue, at the lower pH the mast cells displayed a beautiful violet metachromasia against an as a rule very faintly stained background, although cell nuclei and occasion-

whether such cells contain demonstrable amounts of 5-HT A prelimi nary report of the findings has been published earlier (14)

MATERIALS AND METHODS

Tissue Specimens

This investigation was performed on 5 μ sections of biopsy specimens from car cinoids diagnosed in the Pathological Laboratories Gothenburg in 1952 1964 The series comprised 16 carcinoids of the appendix, 7 of the small bowel and 3 meta stase from primary carcinoids
morphological evidence
of formalin fixed tissue
the present series had been included among the 27 used for a study on the entero chromaffin properties of carcinoid tumour tissue—its argentaffin Schmorl s ferric ferricyanide and alkaline diazo reactions—as reported previously (15) The remain ing 4 carcinoids in the former series had been used up)

At the time of operation only one of the appendiceal carcinoids had given rise to gross evidence of metastatic spread *viz* to the mesentery, omentum and pancreas Clinical signs of excessive serotonin production or of carcinoid syndrome had not been observed in any case

Only 2 of the 7 carcinoids of the small bowel lacked gross metastases at the time of operation In the 5 cases of metastasizing tumours the primary tumour was examined in 3 while metastases to the ovaries and peritoneum were examined in the 2 cases in which no primary tumour was available At the time of the operation 4 of the patients with metastasizing intestinal carcinoid exhibited clinical signs of excessive 5 HT production the peak 24 hour urinary 5 hydroxyindole acetic acid (5 HIAA) excretion in these cases being 520, 80, 70 and 40 mg

The last 3 carcinoids in the series constituted metastases from primary tumours of unknown site In one of these cases a metastasis to a lymph node in the supra clavicular fossa was examined and in the 2 others tissue excised from a tumour focus in the mesenterial root

Sections of normal human appendix were used as control tissue The appendices —5 from males and 5 from females—were checked for absence of histologically manifest abnormalities by selection from the files of the Pathological Laboratories where they were available as paraffin blocks of formalin fixed tissue

Histochemical Procedures

The presence of reducing material was demonstrated with the aid of *Pearse*s adaptation (30) of Schmorl s ferric ferricyanide reaction For a variety of reasons no attempts were made to assess argentaffin reactions First this reaction possesses no theoretical advantages over the ferric ferricyanide reaction Second its specificity is dependent on the duration of the incubation period and the significance of posi tive reactions in stroma cells may be difficult to interpret because these cells are impregnated in a characteristic sequence when the incubation period becomes too long (21) Third in some carcinoids there are discrepancies between the demonstrable argentaffin material on the one hand and ferric ferricyanide reducing and diazo reactive material on the other (15) as well as between demonstrable argentaffin material and extractable 5 HT (39)

The stable diazo compound Fast Garnet GBC and freshly diazotized Fast Red B were used for diazo reactions which were carried out essentially as described by *Lillie* (28) The dyes were Brentamine brand of Fast Garnet GBC and Brentamine brand of Fast Red B base both supplied by courtesy of ICI Ltd Dyestuffs Branch Sweden The coupling times were 3 minutes for the former and 90 seconds for the latter The reactions were performed at pH 8 5 and 8 0 Unlike sections stained with Fast Red B those stained with fast Garnet GBC were also subjected to nuclear stain
As the results obtained by these two
will hereafter be discussed together
ce microscope) was performed on un
orm and mounted in glycerol between
non fluorescent slides and cover glasses The light source an Osram high pressure mercury lamp Type HBO 200, was equipped with filters having a peak transmission

ally the cytoplasm of epithelial cells retained a pale orthochromatic hue Among sections dyed with toluidine blue, some of those stained at pH 4—but none stained at pH 0 6— exhibited metachromasia of the ground substance of the stroma

Staining with "Astra" blue at pH 0 3 disclosed the presence of mast cells with granules of a pure blue colour. The number of cells was approximately the same as in sections stained with toluidine blue at pH 0 6 Staining with toluidine blue at pH 0 3 followed by a rinse in 0 7 N HCl disclosed fewer mast cells with pale violet granules

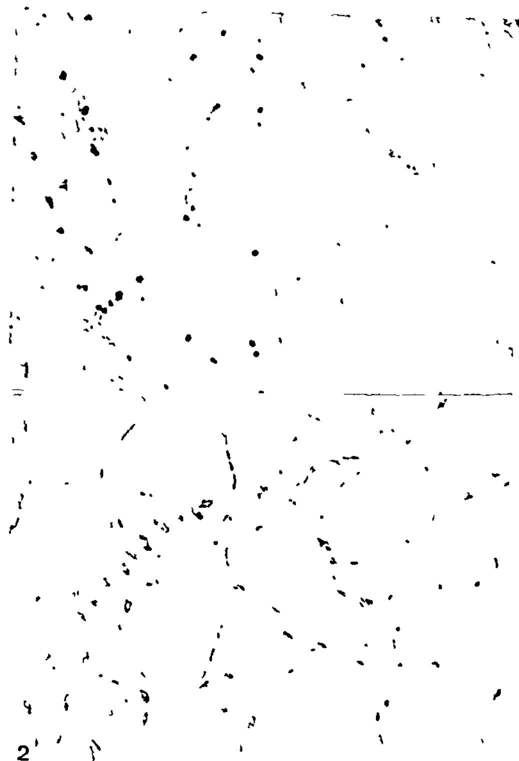
Carcinoid mast cells were usually encountered in the connective tissue stroma, lying along the margins of the tumour lobes like a string of beads, free in the stroma, or around capillaries. Mast cells were also sometimes observed within the tumour lobes, apparently interposed between separate tumour cells The peripheral parts of the tumours were consistently richer in mast cells than the central parts

In intestinal wall from the tumour cases as well as in control appendices, mast cells were encountered at all levels except at the muscular layer Mast cells were most abundant in the submucous layer They occurred in tunica propria mucosae too, but these were most often remarkably weakly stained

The number of mast cells in the tumour stroma varied markedly from one tumour to another Whereas only a single tumour contained a distinctly higher proportion of mast cells than the submucosal layer of a normal appendix, some had approximately the same number as the latter, but several tumours had cross sections with merely a few mast cells Such large variations did not occur in intestinal wall in which the number of submucosal mast cells seemed fairly constant The large variations in carcinoid mast cell numbers were unrelated to the amount of stroma, as tumours with very sparse stroma might contain an abundance of mast cells, and *vice versa*

Fluorescence, Diazo Reactivity and Reducing Properties of Stroma Cells

Inspection of the tumour stroma revealed that in 7 carcinoids this was the site of freely disposed cells containing diazo positive, orange to brick-red granules and of similar cells whose granules exhibited a positive ferric ferricyanide reaction Under the fluorescence microscope these cells emitted a light-yellow fluorescence of the same colour as that of the tumour cells The nuclei appeared as dark, non-fluorescent spots The distribution and morphological appearance of these cells resembled those of mast cells Several ruptured cells were observed with granules scattered around In such cells it was evident that the reactivity was confined to the granules Several carcinoids were characterized by a delicately lobular invasiveness and a tendency to fringing of the tumour lobes In five cases of this type it was difficult to determine whether any reactive, mast cell-like elements were present No free stroma cells



Figs 1 2

Fig 1 Carcinoid with an abundance of mast cells in the connective tissue surrounding the tumour (to the left) The tumour tissue (to the right) is virtually free from mast cells Toluidine blue pH 0 6 × 200

Fig 2 Carcinoid containing a considerable number of mast cells within the tumour tissue The mast cells are situated in the scanty connective tissue stroma between the tumour cell lobes Toluidine blue pH 0 6 × 200

TABLE 1

Data on Carcinoid Tumours Containing Fluorescent Mast Cells

Carcinoid no		File no	Primary site	Metastasis	Stroma mast cells				Tumour cells		
					Cell quantity*	Reactivity†			Reactivity†		
						Diazo	Schmorl	Fluorescence	Diazo	Schmorl	Flourescence
1	P	5178/57	Appendix	Absent	+	+	+	+	++	++	+++
2	P	33/60	Appendix	Absent	++	+	++	++	(+)	+	+
3	P	3164/59	Appendix	Present	++	+	+	+	+	+	+++
4	R	2563/58	Appendix	Absent	+	+	+	+	++	++	++
5		2172/58	Unknown	Present	(+)	+	+	+	++	++	++
6		5056/64	Small Bowel	Present	++	+	+	+	++	++	++
7		6419/64	Small Bowel	Present	++	+	+	+	++	++	+++

* Arbitrarily graded in sections stained with toluidine blue at pH 0.6 (+) = occasional mast cells in whole section + = Few ++ = Intermediate +++ = Abundant (approximately the number in submucosal coat of normal appendix)

† Arbitrarily graded according to intensity of colour and amount of reactive cells (+) = few reactive cells, barely visible, + = weak reaction ++ = intermediate reaction +++ = Strong reaction most cells stained or fluorescent

with positive enterochromaffin reactions were encountered in the remaining 14 carcinoids

No fluorescent, diazo positive or reducing material was observed in the cytoplasm of any cells in intestinal stroma outside the carcinoids or among the abundance of mast cells in the submucous layer of the control appendices Conversely, much fluorescent material occurred in tunica propria mucosae of all appendices as well as in the intestinal mucosa investing the tumours The colour of this fluorescence was more deeply yellow than that of the enterochromaffin cells and bordered on orange when the material was abundant It occurred as conglomerations of coarse granules measuring up to 1 μ in diameter and usually seemed to be intracellular Reactive material having similar morphological characteristics could be demonstrated in sections subjected to the ferric ferricyanide procedure, but direct comparisons revealed that merely a small proportion of the fluorescent material exhibited a positive ferric ferricyanide reaction The fluorescent material had a yellowish brown colour in unstained sections The diazo reaction was always negative, but some diazo stained sections contained abundant pigment of a darker yellow colour than the background It was assumed that this was due to an additive effect of the unspecific yellow background colour and the hue of the pigment Red or orange colour, as of enterochromaffin material, was not encountered in any case In order better to characterize the pigment, some sections were subjected to the periodic acid-Schiff reaction and stained with Sudan Black B The PAS reaction was distinctly positive in all cases, as was the Sudan Black B reaction of the section in which the pigment was most abundant All other sections were negative to Sudan Black B

This pigment in normal intestinal mucosa, where it seems to be accumulated in macrophages, exhibits properties which agree with the so-called pseudomelanin, a substance possessing histochemical characteristics suggesting that it is a lipo-pigment (30) Compared to the cells encountered in the tumour stroma, these pigment charged macrophages had a different morphological appearance, a darker yellow fluorescence and lacked diazo reactivity

Identification of Fluorescent Interstitial Cells in Carcinoids

In an attempt to identify such fluorescent interstitial cells as are encountered in the tumour stroma fluorescence photomicrographs were made of a number of fields from tumours that at the primary examination had been found to contain interstitial cells resembling mast cells and also from tumours whose mode of growth rendered the presence of such cells inconclusive, altogether 12 carcinoids Moreover, corresponding fluorescence photomicrographs were made of fields from tunica propria mucosae of the small bowel and of the appendix containing macrophages with fluorescent pigment granules



4a

3b

4b

3c

4c

The sections were then stained with toluidine blue at pH 0 6 and occasionally also according to May-Grunwald Giemsa. The same fields were sought out and photographed in ordinary transmitted light Thus the interstitial cells with fluorescent material in the *cytoplasm* previously demonstrated in the 7 carcinoids were found actually to be mast cells The sections were thereupon destained with acidified alcohol and checked to see that the mast cells really were microscopically colourless *Following* equilibration with a phosphate buffer at pH 8 for 10 min the diazo reaction was performed on sections from five cases The fluorescent mast cells in all these carcinoids were found to exhibit diazo reactivity Although this reaction was not as strong as in many of the cells observed previously, the cells did aquire a distinctly orange colour In one of the tumours the ferric ferricyanide reaction was performed after extraction of the toluidine blue A resulting sequence of photomicrographs is seen in Fig 3 and 4 No fluorescence localized to mast cells was noted in the tunica propria of the intestinal mucosa

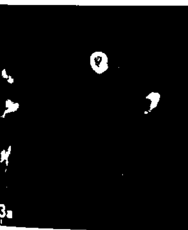
Table 1 presents additional information on such carcinoids as contain fluorescent, diazo positive and ferric ferricyanide reactive mast cells No attempt was made to estimate the proportion of the total mast cell population in the individual carcinoids showing positive reactions It was evident, however, that only a small proportion of all the mast cells were reactive in most carcinoids Carcinoid No 2 differed in some respects from the others It contained a considerable number of mast cells and most of these were fluorescent, diazo reactive and had reducing properties, Fig 3–6 By coincidence it was the tumour cells of this carcinoid which exhibited the weakest reactions, the diazo reaction being barely visible Besides, these tumour cells showed a negative argentaffin reaction Discrepancies between the argentaffin reaction on the one hand and the diazo reactions and the ferric ferricyanide response on the other have been considered in a previous publication (15) The argentaffin reaction had been applied to 5 of the 7 carcinoids containing reactive mast cells and turned out o be positive in 3 and negative in 2 Altogether 6 of the 27 diazo positive carcinoids described previously, were anargentaffin

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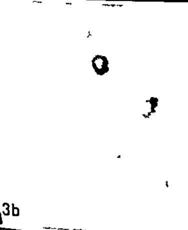
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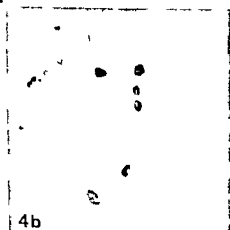
Fig 3 Three photomicrographs from identical fields containing mast cells with fluorescent and reducing cytoplasm Weakly fluorescent tumour lobe to the left × 580 a) Fluorescence photomicrograph b) Toluidine blue pH 0 6 c) Schmorl s ferric ferricyanide reaction

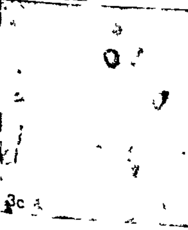
Fig 4 Three photomic
tumour lobes w
cent and reduci
Toluidine blue pH 0 6 c) Schmorl s ferric ferricyanide reaction


3a


4a


3b


4b


3c

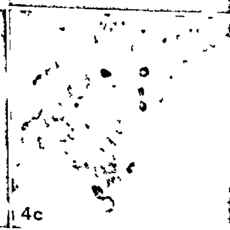
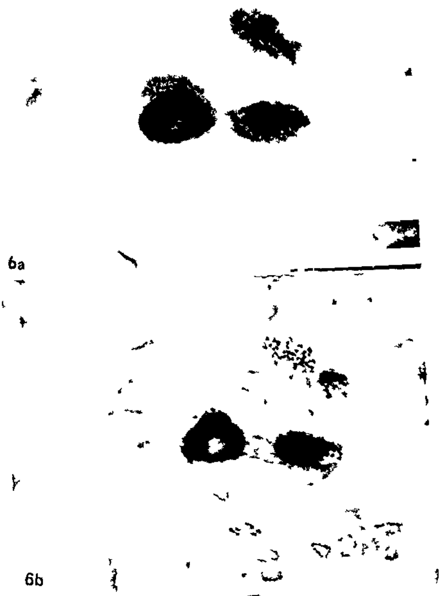

4c



Fig 5

Hight power photomicrograph demonstrating fluorescent mast cells, Approximately × 2,000, a) Fluorescence photomicrograph b) May-Grunwald-Giemza



F g 6

Flu r sc nt na t ell h gh p er ph tom rographs N te the ruptured mast cell w th cattered g anule n F g 6 1 Approx matel × 2 000
a Flu re cen e photon crog aph ( ) May Grunwald G emza

Fluorescence, diazo positiveness and reducing properties could be demonstrated exclusively in mast cells within or near the tumour tissue itself, not in other parts of the tumour containing tissue blocks, such as intestinal wall In the case of carcinoid 7, pieces of small bowel about 10 cm from the primary tumour were taken from the gut resected at the operation and examined So were samples of omentum No reactive mast cells could be demonstrated in either tissue Postoperatively the patient had residual metastases to the liver and a persistently high 5-HIAA excretion On the 11th day after the operation a small biopsy specimen was excised under local anesthesia from the skin on the ventral aspect of the thigh and at once fixed in 10 per cent formalin A comparatively large number of mast cells was demonstrated in the skin specimen but none of these cells displayed fluorescence, diazo positiveness or ferric ferricyanide reactivity During the 24 hours preceding skin biopsy the patient's urinary 5-HIAA output had been 188 mg

The proportion of carcinoids with metastases recorded in Table 1 is somewhat higher than might be expected from the distribution of the whole series Notably, however, the carcinoid containing the highest number of fluorescent mast cells was a non-metastasizing tumour of the appendix The carcinoids containing fluorescent mast cells displayed no additional morphological features distinguishing them from other carcinoids

DISCUSSION

Mast cells have been defined as connective tissue cells that stain metachromatically with thiazine dyes such as toluidine blue (29) Other properties which distinguish mast cell granules from various other metachromatic structures are their alcohol-resistant metachromasia and their persistent staining in strongly acid environments These criteria were met by the cells encountered in the carcinoid stroma

The existence of mast cells in carcinoids is hardly surprising for various other human tumours have been reported to contain mast cells Most important, however, is that some mast cells in formalin-fixed carcinoids exhibit yellow fluorescence in ultraviolet light, reducing properties and alkaline diazo reactivity The only sites in the human organism where this combination of histochemical reactions after formalin fixation has been demonstrated so far are enterochromaffin cells and carcinoid tumour cells In this context it is interesting that after fixation in aqueous formalin solution, chromaffin cells in adrenal medulla exhibit greenish fluorescence and reducing properties but no diazo reactivity (13)

The weight of evidence suggests that the substance which occurs in enterochromaffin cells and carcinoid cells and is responsible for the afore-mentioned reactions is 5-hydroxytryptamine Thus it has been shown that 5-HT is abundant in tissues containing large numbers of

enterochromaffin cells (16, 17); 5-HT has been isolated from carcinoid tumours (27), it has been shown that the reserpine induced fall in intestinal 5 HT content is proportionate to a reduction of histochemical reactivity of enterochromaffin cells (6), it has been demonstrated in model experiments that 5-HT and formaldehyde form a condensation product possessing fluorescence, reducing properties and diazo reactivity (2, 3, 6, 23) It has been adduced that the condensation product of formaldehyde and 5 HT is of the betacarboline type A method recently developed for the demonstration of monoamines on the cellular level (18, 19) is based upon the discovery that the formation of such fluorescent products is facilitated by exposing freeze dried tissue to formaldehyde gas at elevated temperatures This procedure causes 5-HT to yield a 3,4-dihydro-beta carboline with yellow fluorescence and catechol amines to yield 3,4-dihydro-isoquinolines with green fluorescence (11) The superiority of this method for demonstrating 5 HT over the ordinary one involving fixation in formalin solution is evident from the fact that normal mouse and rat mast cells known to contain 5 HT lack fluorescence following fixation in formalin solution but display an intense yellow fluorescence in freeze dried tissue exposed to formaldehyde gas (18) This indicates that the mast cell 5-HT is extractable and that significant amounts of the amine are lost during the conventional fixation and processing techniques Even if all mast cells in the carcinoids contained 5 HT, only those cells which contained the largest initial concentration or bound the amine most avidly would be positive histochemically This could be the reason why only a minor proportion of mast cells in the tumours displayed positive histochemical reactions Similarly the abscence of fluorescence, diazo reactivity and reducing properties in the mast cells of intestinal wall—where the 5-HT content normally is high (16)—and in human skin (13) could mean either that these mast cells do not contain 5-HT at all or that they contain the amine in quantities insufficient to be detected histochemically

The two principal modes by wich 5-HT could become localized to mast cells are by synthesis or specific uptake The synthesis of 5-HT occurs in two principal steps First tryptophan is hydroxylated to 5-hydroxytryptophan (5-HTP), than 5-HTP is decarboxylated to 5-HT (40) 5 HTP decarboxylase activity has been found in different tissues and has also been detected in isolated mast cells from the rat (26) The specific hydroxylating enzyme has, however, a very limited distribution (16) This fact together with the difficulties involved in the finding of demonstrable amounts of 5-HTP in mammalian tissues has led to the conclusion that the hydroxylating reaction is rate-limiting in the synthesis of 5 HT (16) Cases have been reported in which the presence of a carcinoid tumour was attended by a urinary 5 HTP excretion (37) In addition neoplastic mice mast cells in tissue culture have been found capable of synthesizing 5 HT from tryptophan (22), 5-HTP could however not be detected in the mast cells (22) The histochemical reactions

indicative of 5-HT have only been encountered in those mast cells which were situated in close proximity to the tumour cells This makes it likely that the 5-HT in the mast cells is either taken up as 5 HT formed by the tumour cells or as 5-HTP formed by the tumour cells and decarboxylated in the mast cells

It is generally considered that only mast cells in rats and mice contain 5-HT and that human mast cells lack the ability to store this amine (16) The findings reported here have however provided evidence that human mast cells in conditions of enhanced 5-HT production may contain 5-HT

SUMMARY

The connective tissue stroma of carcinoid tumours contains a variable number of mast cells, identifiable by basic dye binding and meta chromasia persisting below pH 1 Unlike mast cells in intestinal wall, some of the carcinoid mast cells have cytoplasmic granules displaying yellow fluorescence in ultraviolet light, reducing properties and alkaline diazo reactivity These reactions are shared with carcinoid tumour cells and enterochromaffin cells and are in all probability due to the presence of 5-hydroxytryptamine (5-HT) This indicates that human mast cells in conditions of enhanced 5-HT formation can concentrate this amine

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Production of Chalone

The skins from 10 mice were utilized for the preparation of each batch of chalone The animals were killed by neck extension and the skin was immediately removed Epidermis was cut off by means of a *Castroviejo Keratotome* In this way it was possible to obtain flat pieces of skin containing the whole epidermis and a little of the upper part of the corium From each skin we could take 6 to 8 pieces of about 1 by 8 cm These were immediately frozen down to minus 70° C by solid carbon dioxide

When a sufficient amount of epidermis had been accumulated, the epidermal pieces were transferred to a porcelain mortar into which liquid air was added This made the skin very brittle thus making it possible to grind the epidermis to a fine, white powder Microscopical control revealed that very few unbroken cells remained after this procedure

The ground epidermis was then transferred to a test tube and about 16 ml of saline was added Further homogenization was performed by an *Elvehjem Potter* homogenizator

Mitosis Counts

The skins were fixed in Bouin s solution After paraffine embedding sections from
Celestine Blue
as counted in 50 microscope "vision
ir 8 Care was taken to count only
ision fields" with an almost straight
as the high and often irregular mitotic
nds were avoided
s about 20 basal cells, or (when
This means that 50 vision fields
nucleated cells

TABLE 1

Mitotic Counts at Different Time Intervals after Various Experimental Procedures

Treatment	Time in hours after first injection	No of animals	Mean no of mitoses per vision field
A Injection with 0 1 mg Colcemid at 0900 a m	1 2 3 4	4 4 4 44	8 5 ± 0 9 18 0 ± 4 4 25 0 ± 2 1 34 8 ± 4 9
B Injection with 0 1 mg Colcemid at 0900 a m and 2 ml Chalone solution 30 minutes later	1 5 2 5 3 5 4 5	5 8 8 8	8 8 ± 1 4 14 0 ± 3 3 16 1 ± 1 2 22 5 ± 2 7
C Injection with 0 1 mg Colcemid at 0900 a m and 2 ml saline 30 minutes later	4 5	8	36 4 ± 3 3
D Injection with 2 ml Chalone solution at 0900 a m	1 5 3 5	8 8	3 8 4 6 } 4 2 ± 0 5
E No injection Mice killed at 0900 a m	-	80	8 5 ± 1 0

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THE EFFECT OF AN EPIDERMIS-SPECIFIC MITOTIC INHIBITOR (CHALONE) EXTRACTED FROM EPIDERMAL CELLS

By

O. H. IVERSEN, E. AANDAHL and K. ELGJO

Received 15.iii.65

The regulation of the mitotic rate in the epidermis has been the subject of much discussion. During the last decade definite progress has been made, especially through the work of Bullough and his school (for a review, see *Bullough & Laurence* 1964 a, *Bullough & Rytomaa* 1965).

It can be concluded from a series of observations (see, for instance *Bullough & Laurence*, 1960, *Oehlert & Buchner* 1961, *Iversen & Evensen* 1962) that the regulation mechanism must be of cybernetic nature based on the presence of a mitotic inhibitor produced by the maturing cells in the epidermis.

Bullough, Hewett & Laurence (1964) have succeeded in extracting such inhibitor from epidermal tissue. They have to a certain extent determined its nature and mode of action. The mitotic inhibitor has been called a *chalone*. According to *Bullough* the substance acts by forming an active complex with adrenaline.

On the basis of studies within experimental carcinogenesis we have found that the reaction of the epidermis after a single application of methylcholanthrene is most adequately explained by a cybernetic theory based on the chalone-effect (see *Iversen & Bjerknes* 1963). We were thus interested in testing the chalone on the strain of hairless mice of this institute.

As *Bullough & Laurence* (1964 b) have even published data concerning the effects of the epidermal chalone on the mitotic *duration*, we have also determined this parameter.

MATERIALS AND METHODS

Hairless mice of the strain hr/hr were used both for the production of epidermal chalone and for the testing of its effect. The mice were about 12 weeks old. All experiments were started in the morning at about 0900 a.m. to avoid as far as possible variations due to the circadian rhythm of the mitotic count. Equal numbers of males and females were used in each experimental group.

... [illegible] standard deviation of 8 71 This means that the mitotic ... from 0900 ... hour per 50 vision fields In per cent of all nucleated cells this is 0 [illegible] mitosis per hour

The Normal Mitotic Duration (Table 1 A, and E)

The mitotic count without any Colcemid injection was measured at 0900 a m in 80 animals The average was found to be 8 5 mitosis per 50 vision fields The mitotic duration can then be calculated.

$$\text{Mitotic duration} = \frac{8\ 5}{8\ 5} = 1 \text{ hour}$$

The Mitotic Rate after Chalone Injection (Table 1 B)

Mice were first injected with Colcemid, and 30 minutes later with 2 ml of the chalone-solution

The line of best fit had the following value

$$Y = 1\ 87 + 4\ 47 \quad X,$$

with a residual standard deviation of 6 44 This means that the mitotic rate in the epidermis of our hairless mice under the influence of the chalone, was reduced to 4 5 mitoses per hour per 50 vision fields In per cent of all cells this is 0 30 mitoses per hour

A statistical calculation (t test) showed a highly significant difference between the normal mitotic rate and the reduced mitotic rate ($p<0\ 005$)

The Mitotic Duration after Chalone Injection (Table 1 B and D)

The mitotic count without Colcemid injection was measured at 1 5 and 3 5 hours after Chalone injection The average mitotic count was found to be 4 2 per vision fields The mitotic duration can then be calculated to

$$\text{Mitotic duration} = \frac{4\ 2}{4\ 5} = 0\ 93 \text{ hour}$$

Control The Mitotic Rate after Saline Injection (Table 1 C)

Mice were first injected with Colcemid, and 30 minutes later with 2 ml of saline The mitotic count 4 hours after the saline injection was found to be 36 4 per 50 vision fields

There is no significant difference between this value and the corresponding value for the line of best fit for the normal mitotic rate (viz 38 9)

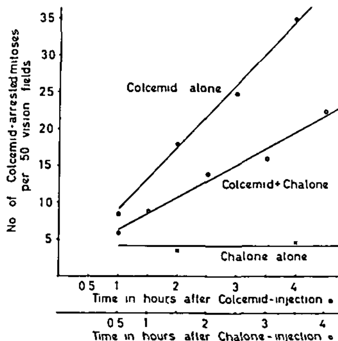


Fig 1

Mitotic counts at different time intervals after injection with Colcemid alone (upper curve), Colcemid plus Chalone (middle curve), and Chalone alone (lower curve)

Measuring the Mitotic Rate

The mitotic rate, *i e* the number of cells entering the mitotic compartment per time unit in a given area of epidermis (50 "vision fields"), was measured by the Colcemid Method Each animal was given 0 1 mg of Colcemid intraperitoneally At regular time intervals during the first 4 5 hours after the Colcemid injection groups of mice (see Table 1) were sacrificed and the mitotic count determined

The line of best fit for the different counts was calculated by the least square method, and the angle between this line and the abscissa was used as the measure of the mitotic rate

Measuring the Mitotic Duration

The experiments were repeated using mice which were not given any Colcemid The mitotic count in 50 "vision fields" from each animal was determined, and the mitotic duration was then calculated from the ratio between the mitotic count and the mitotic rate measured by the Colcemid method, *viz*

$$\text{Mitotic duration} = \frac{\text{Mitotic count (without Colcemid)}}{\text{Mitotic rate (with Colcemid)}}$$

RESULTS

The results are condensed in Table 1 and Fig 1

The Normal Mitotic Rate (Table 1 A)

The calculus for the line of best fit based on these countings had the following value:

$$Y = 0.697 + 8\ 51 \quad X$$

with a residual standard deviation of 8 71 This means that the mitotic rate in the epidermis of our hairless mice during the period from 0900 a m to 0100 p m showed an average of 8 51 mitoses per hour per 50 vision fields In per cent of all nucleated cells this is 0 57 mitosis per hour

The Normal Mitotic Duration (Table 1 A, and E)

The mitotic count without any Colcemid injection was measured at 0900 a m in 80 animals The average was found to be 8 5 mitosis per 50 vision fields The mitotic duration can then be calculated

$$\text{Mitotic duration} = \frac{8\ 5}{8\ 5} = 1 \text{ hour}$$

The Mitotic Rate after Chalone Injection (Table 1 B)

Mice were first injected with Colcemid and 30 minutes later with 2 ml of the chalone solution

The line of best fit had the following value

$$Y = 1\ 87 + 4\ 47\ X$$

with a residual standard deviation of 6 44 This means that the mitotic rate in the epidermis of our hairless mice under the influence of the chalone was reduced to 4 5 mitoses per hour per 50 vision fields In per cent of all cells this is 0 30 mitoses per hour

A statistical calculation (t test) showed a highly significant difference between the normal mitotic rate and the reduced mitotic rate ($p<0\ 005$)

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The mitotic count without Colcemid injection was measured at 1 5 and 3 5 hours after Chalone injection The average mitotic count was found to be 4 2 per vision fields The mitotic duration can then be calculated to

$$\text{Mitotic duration} = \frac{4\ 2}{4\ 5} = 0\ 93 \text{ hour}$$

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Mice were first injected with Colcemid and 30 minutes later with 2 ml of saline The mitotic count 4 hours after the saline injection was found to be 36 4 per 50 vision fields

There is no significant difference between this value and the corresponding value for the line of best fit for the normal mitotic rate (viz 38 9)

SUMMARY AND CONCLUSIONS

Groups of hairless mice (hr/hr) were injected with chalone extracted from epidermal tissue from the same strain of mice The mitotic rate and the mitotic duration were determined by the stathmokinetic method

Under the experimental conditions described above an injection of 2 ml of chalone-solution provoked a significant, and continuous depression of the mitotic rate of the epidermis The depression of the proliferation rate lasted for at least 4 hours The mitotic count was lowered, but in contrast the mitotic duration remained almost unaffected

The possible relationship between our chalone and adrenaline was not investigated

Bullough & Laurence (1964 b) found that their chalone would prolong the mitotic duration As their results were obtained *in vitro* and ours *in vivo*, it seems premature to discuss this problem until further studies have been performed We have even used a very low concentration of chalone as compared with the concentration used by *Bullough & Laurence*

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IMMUNOCHEMICAL STUDIES ON SOME SEROLOGICAL CROSS REACTIONS IN THE *KLEBSIELLA* GROUP

II *Structure of the Capsular Polysaccharides of* Klebsiella aerogenes *Strain B 1076/48 and 'Enterobacter' Strain 349*

By

JORUNN ERIKSEN

Received 28 1 65

Klebsiella aerogenes strain B 1076/48 was isolated from urine, and "Enterobacter" strain 349 from water When growing on lactose bromthymol blue agar, strain B 1076/48 appeared as yellow, moderately mucoid colonies All organisms gave capsular reaction Strain 349 was yellow, flat and transparent Young cultures were non-mucoid After two or three days they became slimy Capsular reaction carried out with strain 349 showed that not all the organisms reacted In fact, most of them seemed to be acapsular The organisms were also *mobile*

Both strains reacted strongly in anti *Klebsiella* type 3(C) serum, but failed to absorb all antibody The capsular polysaccharides of the two strains were isolated by cold water extraction described previously (6) The polysaccharides obtained in this way were used for chemical investigations and as antigens for quantitative precipitation in anti *Klebsiella* type 3(C) sera (4, 7)

The chemical analysis of the polysaccharides isolated from Klebs...

...ucose and rhamnose in addition Chemical investigation of the capsular polysaccharide from *Klebsiella* type 3(C) produced in the same way also showed it to contain uronic acid, mannose and galactose

However the results of the serological investigations were not in agreement with the chemical data Polysaccharide from ... 1076/48 ... *Klebsi*... reacted ...early all of the type specific antibody in spite of the fact that the polysaccharide contained glucose and rhamnose in addition The results could be explained in case the polysaccharide from strain 349 was a mixture of two fractions, one of which reacted with the type 3(C) specific antibody only Evidence for two different antigens has been

SUMMARY AND CONCLUSIONS

Groups of hairless mice (hr/hr) were injected with chalone extracted from epidermal tissue from the same strain of mice. The mitotic rate and the mitotic duration were determined by the stathmokinetic method.

Under the experimental conditions described above an injection of 2 ml of chalone solution provoked a significant, and continuous depression of the mitotic rate of the epidermis. The depression of the proliferation rate lasted for at least 4 hours. The mitotic count was lowered but in contrast the mitotic duration remained almost unaffected.

The possible relationship between our chalone and adrenaline was not investigated.

Bullough & Laurence (1964 b) found that their chalone would prolong the mitotic duration. As their results were obtained *in vitro* and ours *in vivo*, it seems premature to discuss this problem until further studies have been performed. We have even used a very low concentration of chalone as compared with the concentration used by *Bullough & Laurence*.

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Klebsiella aerogenes strain B 1076/48 and "Enterobacter" strain 349 showed that both contained uronic acid, mannose and galactose, but strain 349 contained glucose and rhamnose in addition. Chemical investigation of the capsular polysaccharide from *Klebsiella* type 3(C) produced in the same way also showed it to contain uronic acid, mannose and galactose.

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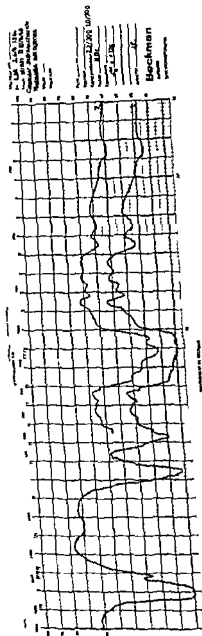


Fig 1

Fig 2

Fig 1 Infra d spectrum of ac d c polysacchar de from *Klebs ella aeroge es* strai B 1 6 48

Fig 2 Inf ar d spe t um of ac d c p l acchar de from Enter bacter strain 34

established by recent investigation (5) of the capsular polysaccharide from *Klebsiella* type 3(C). Polysaccharide obtained by cold water extraction was separated in two different fractions by cetyl *pyridinium* chloride. One fraction was the type specific acidic polysaccharide. The other fraction was a neutral polysaccharide which only reacted in homologous antiserum.

Polysaccharides from strain B 1076/48 and strain 349 were purified in the same way (5). The crude polysaccharide was dissolved in water and the *acidic* polysaccharide was precipitated as a complex by cetyl pyridinium chloride (10). Neither the precipitation of the *acidic* polysaccharide from strain B 1076/48 nor from strain 349 gave a clear endpoint. Consequently the mixture was centrifuged and the supernatant precipitated again till no more complex was formed. Now the supernatant only contained the neutral polysaccharide which was precipitated by 3 volumes of 96 per cent ethanol after concentration to a small volume. By this method two fractions were isolated from each strain, one acidic and one neutral polysaccharide. The chemical and serological reactions of the neutral polysaccharides from the two strains will be dealt with later. Here it shall be mentioned only that the two extra sugars, glucose and rhamnose, which were found in the crude polysaccharide isolated from strain 349, both belonged to the neutral fraction of this strain.

The acidic polysaccharides from both strains reacted with the type specific antibody in *Klebsiella* type 3(C) immune serum, but they were not identical with the type specific polysaccharide.

Phosphorus was determined in both acidic polysaccharides (8) but no phosphorus was present in any of them. Nitrogen was determined by the micro-Kjeldahl method (8). Optical rotation was measured in a Hilger and Watts polarimeter M 412 as Na-salt (c 0.3 water). Table 1 demonstrates the optical rotation and the nitrogen contents of the two strains.

TABLE 1

Optical Rotation and Nitrogen Content of the two Acidic Polysaccharides

Acidic polysaccharides from strain	Optical rotation $[\alpha]_D^{20}$	Per cent nitrogen
Klebsiella aerogenes B 1076/48	+ 125.8°	0.3
Enterobacter 349	+ 117.9°	0.0

The infrared spectra of the two acidic polysaccharides were measured in the same way as for the *Klebsiella* type 3(C) specific polysaccharide in a Beckman I.R. 5A infrared spectrophotometer (5). The two infrared spectra are shown in Fig. 1 and Fig. 2.

This paper is concerned with further investigations of the acidic polysaccharides from *Klebsiella aerogenes* strain B 1076/48 and "Entero-

mannose galactose disaccharide uronic acid and an aldobiuronic acid When the chromatogram was sprayed with silver nitrate sodium hydroxide a new spot appeared which could be a lactone spot

Before cellulose column chromatography the hydrolysate was separated in neutral and acidic sugars The hydrolysate was treated with De-Acidite FF (CO_3) The acidic mono and disaccharides were absorbed by the resin while the neutral sugars remained in the solution The solution was evaporated to dryness and the weight of the neutral sugars was 400 mg The acidic sugars absorbed by the resin, were released again as ammonium salts by treating the De-Acidite FF with 1N ammonium carbonate The ammonium salts were converted to the free acids by Amberlite Resin I R 120 (H) and then the solution was evaporated to dryness The weight of the acidic fraction was 270 mg

The neutral sugars were first separated by column chromatography The glass column used was 450 mm long and the inside diameter was 25 mm The column with a layer of glass fibers in the bottom was packed in the following way

The column was first filled with solvent 2 The cellulose powder was then added as a slurry in the same solvent on the top of the column while the solvent was allowed to run out the bottom The suspension was stirred slowly with a glass rod above the packing line to prevent settling and channelling The column was kept nearly full of slurry at all times When the column had been packed to the desired height the outlet of solvent was stopped and the cellulose powder allowed to set A circle of porous filter paper was put on the top and the column was ready for use The neutral sugars were dissolved in the same solvent and applied to the top of the column Fractions of 10 ml were collected

Three different fractions were isolated which by paper chromatography were identified as mannose galactose and a disaccharide

The mannose fraction had an optical rotation $[\alpha]_D^{0} = + 14\ 2°$ (c 0 5 water) and was characterized as 2 3 5 6 Di O isopropylidene-α D mannofuranose with m p 120 alone and mixed with an authentic sample (3)

The galactose fraction showed an optical rotation $[\alpha]_D^{20} = + 75\ 6°$ (c 0 3 water) and was characterized as β D galactopyranose penta acetate with m p 133° alone and mixed with an authentic sample (11)

The isolated disaccharide was hydrolysed and shown by paper chromatography in solvent 3 to contain mannose and galactose By reduction of the disaccharide with sodium borohydride (5) and hydrolysis of the disaccharide alcohol mannitol and galactose were formed This indicated that the mannose was the reducing end in the disaccharide

The acidic mono and disaccharides isolated from the hydrolysate of the polysaccharide by means of De Acidite FF (CO_3) were separated on a cellulose column in the same way The column was repacked and

bacter" strain 349 The purpose of the investigation is to find similarities and dissimilarities in the structure compared with that of the type specific *Klebsiella* type 3(C) acidic polysaccharide

EXPERIMENTAL

Most of the methods used for structural investigations of acidic *Klebsiella* polysaccharide have been described in detail previously (5) and shall not be repeated in this paper Solvent developers to be used for chromatography were.

1 Butanol ethanol water, 4v 1v 5v
2 Ethylacetate pyridine water 5v 2v 5v
3 Butanol pyridine water, 6v 4v 3v
4 Pyridine ethylacetate glacial acetic acid-water, 5v 5v 1v 3v
5 Butanol ethanol water ammonia, 40v 10v 49v 1v

Partial Hydrolysis of the Acidic Polysaccharides from strain B 1076/48 and Strain 349

A small quantity of polysaccharide was hydrolysed with different concentrations of sulphuric acid for 3 hr at 100° The concentrations used were 2N, 1N, 0 1N and 0 01N By paper chromatography of the hydrolysates in solvent 1 the results were

Strain B 1076/48 hydrolysed with 2N and 1N sulphuric acid Mannose, galactose, uronic acid and aldobiuronic acid The hydrolysates also contained a small amount of a substance with R_f-value between those for galactose and uronic acid This might be a disaccharide It seemed to be most of this substance when the polysaccharide was hydrolysed with 1N sulphuric acid

Strain B 1076/48 hydrolysed with 0 1N sulphuric acid Mannose, galactose and uronic acid But most of the polysaccharide was unhydrolysed

Strain B 1076/48 hydrolysed with 0 01 N sulphuric acid Most of the polysaccharide was unhydrolysed Only a small amount of mannose could be seen on the chromatogram when the paper was sprayed with silver nitrate-sodium hydroxide (5)

Precisely the same results were obtained by hydrolysis of the polysaccharide from strain 349 The same fractions could be seen on the paper chromatograms after hydrolysis with different concentrations of the sulphuric acid

To identify the different fractions of the hydrolysates, 1 g polysaccharide from each strain was hydrolysed for cellulose column chromatography (5) 1N sulphuric acid was used

Cellulose Column Chromatography of the Polysaccharide Hydrolysate from Strain B 1076/48

The weight of the hydrolysate was 750 mg A chromatogram in solvent 2, sprayed with aniline hydrogen phtalate (5) showed it to contain

mannose, galactose, disaccharide, uronic acid and an aldobiuronic acid When the chromatogram was sprayed with silver nitrate-sodium hydroxide, a new spot appeared which could be a lactone spot

Before cellulose column chromatography, the hydrolysate was separated in neutral and acidic sugars The hydrolysate was treated with De-Acidite FF (CO_3) The acidic mono- and disaccharides were absorbed by the resin while the neutral sugars remained in the solution The solution was evaporated to dryness and the weight of the neutral sugars was 400 mg The acidic sugars, absorbed by the resin, were released again as ammonium salts by treating the De-Acidite FF with 1N ammonium carbonate The ammonium salts were converted to the free acids by Amberlite Resin I R 120 (H^+), and then the solution was evaporated to dryness The weight of the acidic fraction was 270 mg

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Three different fractions were isolated which by paper chromatography were identified as mannose, galactose and a disaccharide

The mannose fraction had an optical rotation $[\alpha]_D^{20} = +14.2°$ (c 0 5 water) and was characterized as 2,3 5,6 Di-O-isopropylidene-α-D-mannofuranose with m p 120° alone, and mixed with an authentic sample (9)

The galactose fraction showed an optical rotation $[\alpha]_D^{20} = +75.6°$ (c 0 3 water) and was characterized as β-D-galactopyranose pentaacetate with m p 139° alone, and mixed with an authentic sample (11)

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5 Butanol ethanol water-ammonia, 40v 10v 49v 1v

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Cellulose Column Chromatography of the Polysaccharide Hydrolysate from Strain B 1076/48

The weight of the hydrolysate was 750 mg A chromatogram in solvent 2, sprayed with aniline hydrogen phtalate (5) showed it to contain

nitrate sodium hydroxide These results indicated that most of the uronic acid was galacturonic acid, but also some glucuronic acid was present

5 Aldobiuronic acid which by hydrolysis gave uronic acids and mannose was also isolated The mobility was 0.7 compared with that of galacturonic acid Reduction of the aldobiuronic acid *with lithium* aluminium hydride and hydrolysis of the reduced product, gave galactose mannose and glucose But still the lactone spot could be seen on *the chromatogram* when the paper was sprayed with silver nitrate sodium hydroxide These results indicated two different aldobiuronic acids The main aldobiuronic acid was galacturonic acid mannose Glucuronic acid mannose was also present, but in small amount

Periodate Oxidation of the Acidic Polysaccharide Isolated from Strain B 1076/48 and Strain 349

The polysaccharides were oxidized by sodium periodate *in the dark* at room temperature Aliquots of the reaction mixture were removed periodically and analysed for periodate consumption, formaldehyde and formic acid (5) At the same time mannitol was oxidized as a control Table 2 contains the results of the determinations of the periodate consumption after oxidation of the polysaccharides

TABLE 2

Consumption of Periodate by Oxidation of the Polysaccharides

Time in hours	0.5	2	5	24	48	2
Strain B 1976 48 mol periodate per mol anhydrosugar	0.14	0.28	0.32	0.37		0.37
Strain 349 mol periodate per mol anhydrosugar	0.16	0.26	0.28	0.37	0.37	0.37

At the same time intervals aliquots were drawn for determinations of formaldehyde and formic acid Neither formaldehyde nor formic acid seemed *to be formed during oxidation from any of the strains*

The polysaccharides oxidized for 24 hr from both strains were isolated After hydrolysis paper chromatography of the hydrolysates showed that the *quantity of mannose had decreased during the oxidation, but* still some mannose was left No decrease of galactose or uronic acid could be seen from the chromatograms When the chromatogram of the hydrolysates of the oxidized polysaccharides was sprayed with aniline hydrogen phthalate two strong yellow spots appeared in the uronic acid(s) region of the chromatogram Another yellow spot *appeared* further down on the chromatogram where the lactone spots usually could be seen when the paper was sprayed with silver nitrate-sodium hydroxide These yellow spots appeared at once while the paper was

solvent 4 was used for the packing and the separation Two fractions were isolated and identified by paper chromatography as uronic acid and aldobiuronic acid

The uronic acid showed the same Rf-value as galacturonic acid, and by paper ionophoresis in acetate buffer, pH = 5, the mobility was the same as for hexuronic acid. The isolated uronic acid was converted to the methyl ester methyl glycoside and reduced with lithium aluminium hydride (5) Paper chromatography in solvent 3 after hydrolysis showed a content of mannose when the paper was sprayed with aniline hydrogen phtalate However, if the paper was sprayed with silver nitrate-sodium hydroxide, a second spot appeared This could be a lactone spot The yield of mannose after reduction of the uronic acid was very small which might be due to the presence of lactone The isolated uronic acid seemed to be mannuronic acid

The aldobiuronic acid isolated from the cellulose column had an optical rotation $[\alpha]_D^{20} = +73.0°$ (c 0.3 water) By paper ionophoresis in acetate buffer, pH = 5, the fraction had a mobility of 0.7 compared with galacturonic acid Hydrolysis of the aldobiuronic acid with 2N sulphuric acid for 5 hr and paper chromatography of the hydrolysate in solvent 4, showed a content of uronic acid and mannose, but still some unhydrolysed aldobiuronic acid The aldobiuronic acid was uronic acid linked to mannose

The aldobiuronic acid was then converted to the methyl ester methyl glycoside and reduced with lithium aluminium hydride The hydrolysate of the methyl bioside showed to contain only mannose The aldobiuronic acid was therefore identifed as mannuronic acid linked to mannose

Cellulose Column Chromatography of Polysaccharide Hydrolysate from Strain 349

The weight of the hydrolysate was 800 mg which was applied to the top of the column without being separated in neutral and acidic fractions Solvent 3 was used Five fractions were isolated

1 Mannose, $[\alpha]_D^{20} = +19.5°$ (c 0.4 water), characterized as 2,3 5,6 Di-O-isopropylidene-α-D-mannofuranose with m p 120° alone, and mixed with an authentic sample

2 Galactose, $[\alpha]_D^{20} = +63.5°$ (c 0.4 water), characterized as β D-galactopyranose pentaacetate with m p 139° alone, and mixed with an authentic sample

3 A disaccharide, which was galactose linked to mannose Mannose was the reducing end of the disaccharide

4 Uronic acid, which had the same mobility as galacturonic acid in acetate buffer, pH = 5 By reduction of the uronic acid with lithium aluminium hydride, galactose was isolated Spots of glucose and lactone could be seen on the chromatogram when it was sprayed with silver

main acid was galacturonic acid, but it also contained a small quantity of glucuronic acid The presence of two different uronic acids in the same strain, has been reported earlier (1,2) *Aerobacter aerogenes* NCTC 418 and *Aerobacter aerogenes* NCTC 8172 both contained glucuronic and mannuronic acid Whether the two uronic acids found in strain 349 are units in the same polysaccharide, or in two different polysaccharides, is still a question

During periodate oxidation neither formaldehyde nor formic acid was formed which indicated the absence of hexofuranose units and pyranose units linked 1,6 Since the polysaccharides were not resistent to periodate attack, but showed a consumption of 0 37 mol periodate per mol anhydrosugar, about 1/3 of the linkages must be 1 4 The other linkages could be 1,3 since they were resistant to oxidation, but the

was still some mannose left This indicated that the mannose must be linked both 1,3 and 1,4 A decrease in the quantity of galactose and uronic acid after oxidation could not be seen from the chromatogram The oxidation of the polysaccharides seemed to be finished in less than 24 hr and no overoxidation could be seen The uronic acid could therefore neither be linked through the 2-position nor be the non reducing end group

When the oxidized polysaccharides were hydrolysed, and a chromatogram of the hydrolysates was sprayed with aniline hydrogen phtalate, some yellow spots appeared at once while the paper was still wet When the hydrolysates were treated with De-Acidite FF (CO_3) the spots did not come out The colour must be due to a reaction between aniline hydrogen phtalate and the acidic fraction in the hydrolysate Strain B 1076.48 contained mannuronic acid, and strain 349 contained galacturonic and glucuronic acid Both mannuronic and glucuronic acid easily form lactone, while galacturonic acid does not Galacturonic acid is part of the *Klebsiella* type 3(C) polysaccharide (5) and these yellow spots were never seen on a chromatogram of the oxidized *Klebsiella* type 3(C) hydrolysate

The study of the methylated polysaccharides clearly indicated that the methylation was incomplete Polysaccharides containing a high proportion of uronic acid are often very resistant to complete methylation (3) The chromatograms were difficult to interpret, but in the hydrolysates from both strains 2,4 6 tri-O-methyl-D galactose and 2 3 6 tri O methyl D mannose were found These results indicated that mannose was linked 1,4 and galactose was linked 1,3 Small amounts of 2 3 4 6 tetra-O methyl mannose confirmed that mannose was the non reducing end group in both polysaccharides Mannose was also first released by hydrolysis of the polysaccharides with 0 01N sulphuric acid at 100° for 3 hr

still wet When the hydrolysate beforehand was treated with De-Acidite FF (CO_3^{--}) the yellow spots did not come out The yellow colour must be due to a reaction product of aniline hydrogen phtalate and the oxidized aldobiuronic acids.

Alkali Treatment of the Acidic Polysaccharides Isolated from Strain B. 1076/48 and Strain 349

Before methylation, the polysaccharides were treated with 1N sodium hydroxide in an atmosphere of nitrogen (5) At different intervals aliquots were neutralized and used as antigens in gel precipitations against homologous antisera Alkali had no effect on the polysaccharides as antigens, even after 3 days No difference could be seen before and after treatment by alkali, and the results were the same for both strains

Methylation of the Acidic Polysaccharides from Strain B 1076/48 and Strain 349

Since the polysaccharides were stabile against alkali, Haworth's method with sodium hydroxide and dimethyl sulphate was used for the methylation (5) The methylation was repeated four times The methylated product was hydrolysed (5) and the hydrolysate was applied to a paper chromatogram in solvent 5 for 17 hr When sprayed with aniline hydrogen phtalate, several spots appeared, and it was difficult to interpret the chromatogram The methylation was incomplete, but a few spots could be identified Compared with standards, 2,4,6-tri-O-methyl-D-galactose and 2,3,6-tri-O-methyl-D-mannose were identified A small quantity of 2,3,4,6-tetra-O-methyl-mannose was also present Mono- and dimethylated sugars were present, but no effort was made to identify them The presence of these sugars must be due to the incomplete methylation which is often the case when the polysaccharide contains uronic acid (3) By methylation there was no difference between the two strains

DISCUSSION

By cetyl pyridinium chloride, the crude polysaccharides isolated from *Klebsiella aerogenes* strain B 1076/48 and "Enterobacter" strain 349 were separated in two fractions, one neutral and one acidic The acidic polysaccharide from both strains was responsible for the cross-reaction with *Klebsiella* type 3(C) The acidic polysaccharides from strain B 1076/48 and strain 349 were investigated in detail Both strains contained D-mannose, D-galactose and a disaccharide which was composed of galactose and mannose Mannose was the reducing end in the disaccharide

The uronic acids were different for the two strains Strain B 1076/48 contained mannuronic acid Strain 349 contained two uronic acids The

main acid was galacturonic acid, but it also contained a small quantity of glucuronic acid The presence of two different uronic acids in the same strain, has been reported earlier (1,2) *Aerobacter aerogenes* NCTC 418 and *Aerobacter aerogenes* NCTC 8172 both contained glucuronic and mannuronic acid Whether the two uronic acids found in strain 349 are units in the same polysaccharide, or in two different polysaccharides, is still a question

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not come out The colour must be due to a reaction between aniline hydrogen phtalate and the acidic fraction in the hydrolysate Strain B 1076 48 contained mannuronic acid, and strain 349 contained galacturonic and glucuronic acid Both mannuronic and glucuronic acid easily form lactone, while galacturonic acid does not Galacturonic acid is part of the *Klebsiella* type 3(C) polysaccharide (5) and these yellow spots were never seen on a chromatogram of the oxidized *Klebsiella* type 3(C) hydrolysate

The study of the methylated polysaccharides clearly indicated that the methylation was incomplete Polysaccharides containing a high proportion of uronic acid are often very resistant to complete methylation (3) The chromatograms were difficult to interpret, but in the hydrolysates from both strains 2,4,6-tri O-methyl D galactose and 2,3 6-tri O methyl D mannose were found These results indicated that mannose was linked 1,4 and galactose was linked 1,3 Small amounts of 2 3,4 6-tetra O methyl mannose confirmed that mannose was the non reducing end group in both polysaccharides Mannose was also first released by hydrolysis of the polysaccharides with 0 01N sulphuric acid at 100° for 3 hr

still wet When the hydrolysate beforehand was treated with De-Acidite FF (CO_3) the yellow spots did not come out The yellow colour must be due to a reaction product of aniline hydrogen phtalate and the oxidized aldobiuronic acids

Alkali Treatment of the Acidic Polysaccharides Isolated from Strain B 1076/48 and Strain 349

Before methylation, the polysaccharides were treated with 1N sodium hydroxide in an atmosphere of nitrogen (5) At different intervals aliquots were neutralized and used as antigens in gel precipitations against homologous antisera Alkali had no effect on the polysaccharides as antigens, even after 3 days No difference could be seen before and after treatment by alkali, and the results were the same for both strains

Methylation of the Acidic Polysaccharides from Strain B 1076/48 and Strain 349

Since the polysaccharides were stabile against alkali, Haworth's method with sodium hydroxide and dimethyl sulphate was used for the methylation (5) The methylation was repeated four times The methylated product was hydrolysed (5) and the hydrolysate was applied to a paper chromatogram in solvent 5 for 17 hr When sprayed with aniline hydrogen phtalate, several spots appeared, and it was difficult to interpret the chromatogram The methylation was incomplete, but a few spots could be identified Compared with standards, 2,4,6-tri O-methyl-D-galactose and 2,3,6-tri-O-methyl-D-mannose were identified A small quantity of 2,3,4,6-tetra-O methyl-mannose was also present Mono- and dimethylated sugars were present, but no effort was made to identify them The presence of these sugars must be due to the incomplete methylation which is often the case when the polysaccharide contains uronic acid (3) By methylation there was no difference between the two strains

DISCUSSION

By cetyl pyridinium chloride, the crude polysaccharides isolated from *Klebsiella aerogenes* strain B 1076/48 and "Enterobacter strain 349 were separated in two fractions, one neutral and one acidic The acidic polysaccharide from both strains was responsible for the cross-reaction with *Klebsiella* type 3(C) The acidic polysaccharides from strain B 1076/48 and strain 349 were investigated in detail Both strains contained D-mannose, D-galactose and a disaccharide which was composed of galactose and mannose Mannose was the reducing end in the disaccharide

The uronic acids were different for the two strains Strain B 1076/48 contained mannuronic acid Strain 349 contained two uronic acids The

6 *Henriksen S D & Eriksen J* Immunochemical studies on some serological cross reactions in the *Klebsiella* group 3 Further studies on the cross reactions between types A(1) E(5) and AE Acta path et microbiol scandinav 51 259 274 1961
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However, further interpretation of the hydrolysates of the methylated polysaccharides was difficult because of incomplete methylation

The high specific rotation of the polysaccharides from strain B 1076/48 (+ 125 8°) and strain 349 (+ 117 9°) indicated α-linkages

SUMMARY

Capsular polysaccharides of *Klebsiella aerogenes* strain B 1076/48 and "Enterobacter" strain 349 were purified and investigated

Klebsiella aerogenes strain B 1076/48 contained

1 D-mannose, D-galactose and mannuronic acid
2 The non reducing end group was mannose
3 The mannuronic acid was linked to mannose as an aldobiuronic acid
4 A disaccharide was isolated which showed to be galactose 1 – mannose 4 (or 3)
5 Mannose was linked both in 3 and 4 position
6 Galactose was linked in 3-position

"Enterobacter" strain 349 contained

1 D-mannose, D-galactose, galacturonic and glucuronic acid
2 The non reducing end group was mannose
3 The uronic acids were linked to mannose as aldobiuronic acids
4 A disaccharide was isolated which showed to be galactose 1 mannose 4 (or 3)
5 Mannose was linked both in 3 and 4 position
6 Galactose was linked in 3-position

A comparison of the structure of the capsular polysaccharides from *Klebsiella aerogenes* strain B 1076/48, "Enterobacter" strain 349 and *Klebsiella type 3(C)*, shows that the only demonstrated difference between them is the nature of the uronic acids

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in ... Antisera against the six
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RESULTS

The results of the quantitative precipitin determinations in antiserum against *Klebsiella ozaenae* type 3(C) strain 3828/60 are shown in Table 1. Acidic polysaccharides from the same sero-type 3(C), but from four different species, were used as antigens.

TABLE 1

Quantitative Precipitin Determinations in Immune Serum against Klebsiella ozaenae *Type 3(C) Strain 3828/60*

Microgram antigen added	Microgram antibody N precipitated in 1 ml serum with polysaccharides from strain			
	3828/60	F 10 N Y	92/04	M A 73
50	358	361	358	356
75	423	–		–
100	465	454	456	442
150	504	509	504	507
200	496	501	490	486
250	490	495	487	485

The same antigens were used for the quantitative precipitation in antiserum against *Klebsiella pneumoniae* type 3(C) strain F 10 N Y. The results are shown in Table 2.

Table 3 shows the results of the quantitative precipitations in antiserum against *Klebsiella rhinoscleromatis* type 3(C) strain 92/04. The same acidic polysaccharides were used as antigens.

The last quantitative precipitin determinations were carried out in antiserum against *Aerobacter* (*Klebsiella*) *aerogenes* type 3(C) strain M A 73. The same antigens were used. The results are shown in Table 4.

From the quantitative precipitin determinations in immune sera against the four different species, all of the sero-type 3(C), the capsular acidic polysaccharides precipitated the same quantity of antibodies

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IMMUNOCHEMICAL STUDIES ON SOME SEROLOGICAL CROSS-REACTIONS IN THE *KLEBSIELLA* GROUP

12. *Serological Reactions with the Acidic Capsular Polysaccharides from* Klebsiella *Type 3(C) as Antigen*

By

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Received 27.iii.65

The capsular acidic polysaccharides of the different species, *Klebsiella ozaenae* (strain 3828/60), *Klebsiella pneumoniae* (strain F 10 N.Y.), *Klebsiella rhinoscleromatis* (strain 92/04) and *Aerobacter (Klebsiella) aerogenes* (strain M.A. 73), all belonging to sero-type *3(C)*, were by chemical methods suggested to be identical (1). Serological investigations (4) with polysaccharides from the same strains, extracted by the cold water method (3), have been described earlier. Quantitative precipitin determination in immune sera with homologous polysaccharide as antigen, showed more precipitate than when heterologous polysaccharides were used. This could be explained if the polysaccharide were a mixture of two antigens, one of which only reacted in homologous antiserum. By further purification of the crude polysaccharide two fractions were isolated. One of the fractions was the type specific acidic polysaccharide. The other fraction was a neutral polysaccharide which as antigen only reacted in homologous antiserum.

The purpose of this paper is to investigate the serological reactions of the type 3(C) specific polysaccharides isolated from the four different species.

MATERIAL AND METHODS

Antigens. The capsular acidic polysaccharides isolated from *Klebsiella ozaenae* (strain 3828/60), *Klebsiella pneumoniae* (strain F 10 N.Y.), *Klebsiella rhinoscleromatis* (strain 92/04) and *Aerobacter (Klebsiella) aerogenes* (strain M.A. 73), all type 3(C) were used as antigens. In addition two capsular acidic polysaccharides isolated from *Klebsiella pneumoniae* type 3(C) (strain 270/60 and strain 1204) were used as antigens for the gel precipitations. All six polysaccharides were the same as those used for the chemical investigations (1).

Antiserum. Young rabbits were injected intravenously every fifth day with 0.1-0.3 ml of a 20 hrs broth culture. The first three injections were carried out with formalin killed organisms. After about two months blood was drawn from the heart of the rabbits under ether anaesthesia. The rabbits were given additional injections and were bled repeatedly if the antibody titre was satisfactory. The sera were kept frozen below —20°. Before use, the sera were inactivated at 56° for 30 min. and centrifuged

in the cold for 2 hrs to get rid of any insoluble matter Antisera against the six strains mentioned above were prepared in this way

Quantitative precipitin determinations were carried out in immune sera against the four species by the method of Heidelberger and Kendall as described by *Kabat & Mayer* (5)

RESULTS

The results of the quantitative precipitin determinations in antiserum against *Klebsiella ozaenae* type 3(C) strain 3828 60 are shown in Table 1 Acidic polysaccharides from the same sero type 3(C), but from four different species, were used as antigens

TABLE 1

Quantitative Precipitin Determinations in Immune Serum against Klebsiella ozaenae *Type 3(C) Strain 3828/60*

Microgram antigen added	Microgram antibody N precipitated in 1 ml serum with polysaccharides from strain			
	3828 60	F 10 N Y	92 04	M A 73
50	358	361	358	356
75	423	–	–	–
100	465	454	456	442
150	504	509	504	507
200	496	501	490	488
250	490	495	487	485

The same antigens were used for the quantitative precipitation in antiserum against *Klebsiella pneumoniae* type 3(C) strain F 10 N Y The results are shown in Table 2

Table 3 shows the results of the quantitative precipitations in antiserum against *Klebsiella rhinoscleromatis* type 3(C) strain 92/04 The same acidic polysaccharides were used as antigens

The last quantitative precipitin determinations were carried out in antiserum against *Aerobacter (Klebsiella) aerogenes* type 3(C) strain M A 73 The same antigens were used The results are shown in Table 4

From the quantitative precipitin determinations in immune sera against the four different species all of the sero type 3(C), the capsular acidic polysaccharides precipitated the same quantity of antibodies

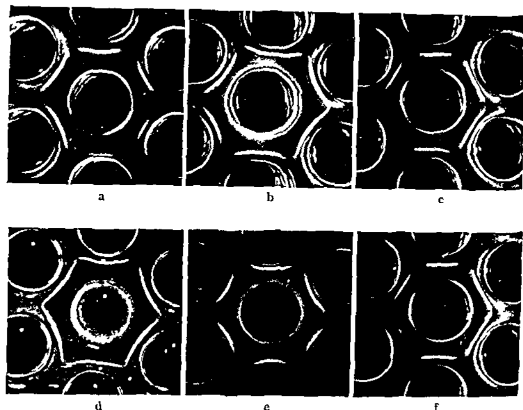


Fig 1

Gel precipitations in immune sera against a *Klebsiella ozaenae* 3828/60 b *Klebsiella pneumoniae* F 10 N Y c *Klebsiella pneumoniae* 1204 d *Klebsiella pneumoniae* 270/60, e *Klebsiella rhinoscleromatis* 92/04 f *Aerobacter (Klebsiella) aerogenes* M A 73, all type 3(C) The capsular acidic polysaccharides from the same strains are placed in the peripheral wells clockwise in the same order

This indicated that the polysaccharides were identical These results are in agreement with the chemical investigations (1)

The results of the gel precipitations are shown in Fig 1 As mentioned earlier in this paper, two more sera and antigens were used for the gel precipitations In all six sera the precipitation lines are identical

TABLE 2

Quantitative Precipitin Determinations in Immune Serum against Klebsiella pneumoniae *Type 3(C) Strain F 10 N Y*

Microgram antigen added	Microgram antibody N precipitated in 0.5 ml serum with polysaccharides from strain			
	F 10N Y	3828/60	92/04	M A 73
100	244	235	244	252
200	283	274	277	269
300	263	258	263	256
400	246	238	246	232
600	210	221	210	202

TABLE 3

Quantitative Precipitin Determinations in Immune Serum against Klebsiella rhinoscleromatis *Type 3(C) Strain 92 04.*

Microgram antigen added	Microgram antibody N precipitated in 0 5 ml serum with polysaccharides from strain			
	92 04	F 10 N Y	3828 60	M A 73
100	384	392	381	392
200	465	440	431	462
300	532	532	532	538
400	552	552	546	534
600	549	538	538	543

TABLE 4

Quantitative Precipitin Determinations in Immune Serum against Aerobacter (Klebsiella) aerogenes *Type 3(C) Strain M A 73*

Microgram antigen added	Microgram antibody N precipitated in 0 5 ml serum with polysaccharides from strain			
	M A 73	F 10 N Y	3828 60	92 04
100	274	266	270	266
200	330	330	330	336
300	367	363	363	356
400	372	372	375	367
600	344	339	339	333

DISCUSSION

Polysaccharides used as antigens in earlier serological investigations (4) were contaminated with a second antigen By purification the antigens could be further separated The results obtained by these serological investigations clearly show that the capsular acidic polysaccharides from the four different species, all of the sero-type 3(C), were identical This is in agreement with the chemical investigation of the same polysaccharides (1)

SUMMARY

The capsular acidic polysaccharides from *Klebsiella ozaenae, Klebsiella pneumoniae, Klebsiella rhinoscleromatis* and *Aerobacter (Klebsiella) aerogenes*, all type 3(C) showed identical serological reactions

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Acta path et microbiol scandinav 64, 527-533, 1965

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IMMUNOCHEMICAL STUDIES ON SOME SEROLOGICAL CROSS REACTIONS IN THE *KLEBSIELLA* GROUP

13 *Serological Investigation of the Cross-Reaction of* Klebsiella *Type 3(C)*, Klebsiella aerogenes *Strain B 1076/48 and "Enterobacter" Strain 349*

By

JORUNN ERIKSEN

Received 27 iii 65

The cross reaction of *Klebsiella type* 3(C), *Klebsiella aerogenes* strain B 1076/48 and *'Enterobacter"* strain 349 has been described earlier in this series (4) Recent investigation (1, 2) showed that the polysaccharides used as antigens for the former examination, were a mixture of two fractions, one neutral and one acidic polysaccharide The acidic fraction was the type specific capsular polysaccharide which was responsible for the cross-reaction between the three strains This paper will deal with the same cross-reaction, but the purified capsular acidic polysaccharides will be used as antigens

MATERIAL AND METHODS

Antigens The capsular acidic polysaccharides from *Klebsiella aerogenes* strain B 1076/48 *Enterobacter* strain 349 and from four strains of *Klebsiella* type 3(C) were used as antigens

lla aerogenes strain

bbits as described in

(3)

RESULTS

The quantitative precipitin determinations in anti-type 3(C) serum are shown in Fig 1 The acidic polysaccharide from strain 349 precipitates 83 per cent of the type 3(C) specific antibodies while strain B 1076/48 *only precipitates 42 per cent* The cross-reaction between *Klebsiella* type 3(C) and *"Enterobacter"* strain 349 is much stronger than the cross reaction between *Klebsiella* type 3(C) and *Klebsiella aerogenes* strain B 1076/48

The results of the quantitative precipitin determinations in antiserum against *"Enterobacter'* strain 349 *are* shown in Fig 2 The type 3(C)

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IMMUNOCHEMICAL STUDIES ON SOME SEROLOGICAL CROSS REACTIONS IN THE *KLEBSIELLA* GROUP

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By

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Received 27.iii.65

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MATERIAL AND METHODS

Antigens. The capsular acidic polysaccharides from *Klebsiella aerogenes* strain B 10 6 48, "*Enterobacter*" strain 349 and from four strains of *Klebsiella* type 3(C) were used as antigens.

-lla aerogenes strain
bbits as described in

(3)

RESULTS

The quantitative precipitin determinations in anti-type 3(C) serum are shown in Fig. 1. The acidic polysaccharide from strain 349 precipitates 83 per cent of the type 3(C) specific antibodies while strain B 1076/48 only precipitates 42 per cent. The cross reaction between *Klebsiella* type 3(C) and *Enterobacter* strain 349 is much stronger

against "*Enterobacter*" strain 349 are shown in Fig. 2. The type 3(C)

3 *Henriksen S D & Eriksen J* Immunochemical studies on some serological cross reactions in the *Klebsiella* group 3 Further studies on the cross reaction between types A(1), E(5) and AE Acta path et microbiol scandinav *51* 259 274, 1961

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aerogenes strain
s as described in

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The quantitative precipitin determinations in anti-type 3(C) serum are shown in Fig 1 The acidic polysaccharide from strain 349 precipitates 83 per cent of the type 3(C) specific antibodies while strain B 1076 48 only precipitates 42 per cent The cross-reaction between *Klebsiella* type 3(C) and '*Enterobacter*" strain 349 is much stronger than the cross reaction between *Klebsiella* type 3(C) and *Klebsiella aerogenes* strain B 1076 48

The results of the quantitative precipitin determinations in antiserum against "*Enterobacter*' strain 349 are shown in Fig 2 The type 3(C)

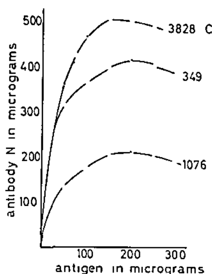


Fig 1

Precipitin curves in immune serum against *Klebsiella ozaeneae* type 3(C) strain 3828//60 The curves show micrograms antibody N per ml serum

specific polysaccharide precipitates 83 per cent of the quantity precipitated by the homologous capsular polysaccharide Polysaccharide from strain B 1076/48 removes only 41 per cent of the antibodies

As shown in Fig 3, the cross-reaction between strain B 1076/48 and strain 349 is nearly the same as that between strain B 1076/48 and *Klebsiella* type 3(C) The difference is only 5 per cent

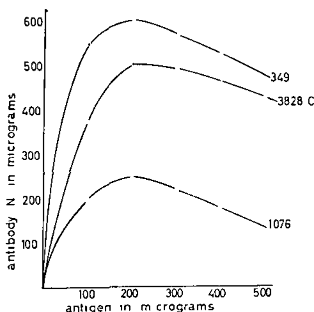


Fig 2

Precipitin curves in immune serum against *Enterobacter* strain 349
The curves show micrograms antibody N per 0 5 ml serum

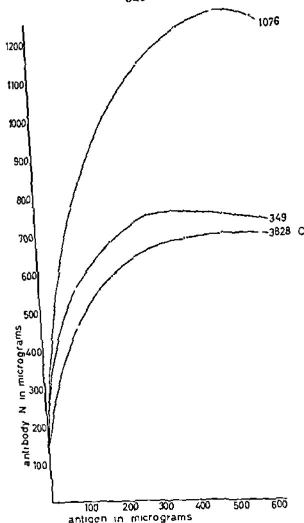


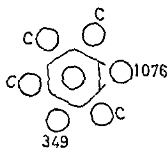
Fig 3

Precipitin curves in immune serum against *Klebsiella aerogenes* strain B 1076/48
The curves show micrograms antibody N per ml serum

Gel precipitation in anti type 3(C) serum is shown in Fig 4 By this
lysaccharide from strain
gens The cross-reaction
[illegible]

Fig 5 and Fig 6 show the gel precipitation in antiserum against Enterobacter" strain 349 The results are in agreement with the quantitative precipitin determinations (Fig 2) In both figures the difference between capsular antigen from *Klebsiella* type 3(C), *Klebsiella aerogenes* strain B 1076 48 and "*Enterobacter*" strain 349 are demonstrated.

Gel precipitation in antiserum against *Klebsiella aerogenes* strain



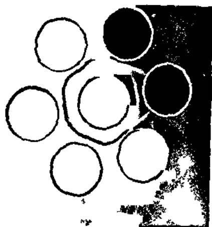
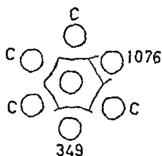


Fig 4
Gel precipitation Immune serum against *Klebsiella* type 3(C) in central well



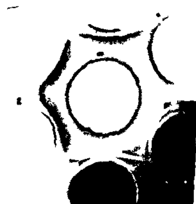


Fig 5

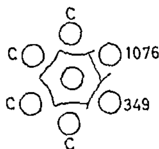




Fig 6
Fig 5 and *Fig* 6 Gel precipitation Immune serum against *Enterobacter* strain 349 in central well

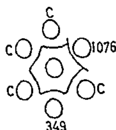
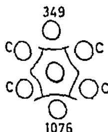




Fig 7



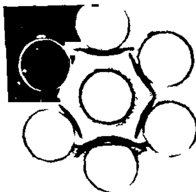


Fig 8

Fig 7 and *Fig* 8 Gel precipitation Immune serum against *Klebsiella aerogenes* strain B 1076/48 in central well The dish in Fig 8 has been left for about one week

B 1076 48 is shown in Fig 7 There again no difference can be seen between strain 349 and type 3(C) polysaccharides as antigens However, on a gel precipitation dish left as long as one week, the difference between the two strains can be demonstrated This is shown in Fig 8

DISCUSSION

A comparison of the two methods, the quantitative precipitin determination and the gel precipitation shows that the former is more sensitive when polysaccharides are used as antigens Small differences between the antigens can easily be missed by gel precipitation For this reason the cross reactions between *Klebsiella* type 3(C), *Klebsiella aerogenes* strain B 1076 48 and '*Enterobacter*' strain 349 are discussed after the results obtained by the quantitative precipitin determinations

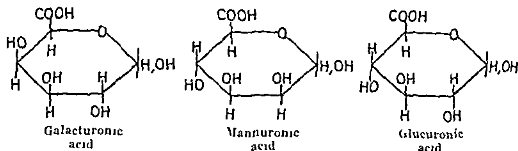


Fig 9

The structure of the three uronic acids present in the capsular polysaccharides

Recent chemical investigations (1, 2) have shown that the three capsular acidic polysaccharides mentioned above resemble one another in their content of mannose and galactose They also seemed to be built in the same way The only demonstrable difference in the structure of the three polysaccharides, was the nature of the uronic acid *Klebsiella* type 3(C) specific polysaccharide contains galacturonic acid Capsular polysaccharide from "*Enterobacter*" strain 349 contains galacturonic acid as the main hexuronic acid, but it also contains a small amount of glucuronic acid Capsular polysaccharide from *Klebsiella aerogenes* strain B 1076/48 contains mannuronic acid The structure of the three uronic acids is described in Fig 9

The cross-reaction between *Klebsiella* type 3(C) and "*Enterobacter* strain 349 must be due to the fact that the capsular polysaccharide in both strains contain galacturonic acid The type 3(C) specific polysaccharide precipitates 83 per cent of the antibodies in anti strain 349 serum, and the capsular polysaccharide from strain 349 precipitates 82 per cent of the antibodies in anti *Klebsiella* type 3(C) serum The influence on the specificity due to the small content of glucuronic acid in the polysaccharide from strain 349 is clearly demonstrated

The capsular polysaccharide from *Klebsiella* strain B 1076/48 precipitates the same quantity of antibodies from anti *Klebsiella* type 3(C) serum and from anti strain 349 serum (42 and 41 per cent) This must be due to the difference in the structure of galacturonic- and mannuronic acid

In antiserum against *Klebsiella aerogenes* strain B 1076/48, the polysaccharide from strain 349 precipitates about 5 per cent more antibodies than the type 3(C) specific polysaccharide This small difference seems to be due to the content of glucuronic acid in the polysaccharide from strain 349 A comparison of the structure of the three uronic acids reveals a greater similarity between mannuronic- and glucuronic acid than between mannuronic- and galacturonic acid

SUMMARY

The cross-reactions between *Klebsiella* type 3(C), *Klebsiella aerogenes* strain B 1076/48 and "*Enterobacter*" strain 349 have been investi-

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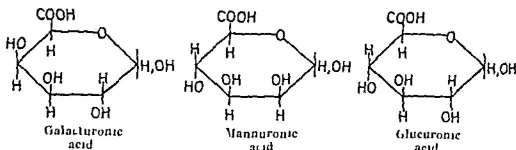


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Acta path et microbiol scandinav 64, 534–542, 1965

The National Institute of Public Health, Virus Department B, Oslo, Norway
(Head A Harboe, M D)

A URONIC AND SIALIC ACID FREE CHICK ALLANTOIC MUCOPOLYSACCHARIDE SULPHATE WHICH COMBINES WITH INFLUENZA VIRUS HI-ANTIBODY TO HOST MATERIAL

1 *Purification of the Substance*

By

GUNNAR HAUKENES, ARILD HARBOE and
KAREN MORTENSSON-EGNUND

Received 15 ii 65

Previous investigations have shown that rabbits injected with normal chick or turkey allantoic materials produce antibodies which inhibit the haemagglutination of influenza virus propagated in the chick, respectively the turkey, chorioallantois (7, 10) Influenza virus grown in other tissues was not inhibited (8, 11) The haemagglutination inhibition by antiserum to allantoic material was blocked when allantoic fluid was added to the serum (8)

The chemical properties of the antigen in the allantoic fluid have been studied, and it was found to be a mucopolysaccharide (8, 15) Since then, these studies have been extended, and the present paper describes an improved purification of the antigen concerned

MATERIALS AND METHODS

The *testvirus* (a red cell eluate of the influenza B strain Lee) and *antiserum* (against chick allantoic material) were prepared as described in earlier publications (7, 8)

The HIB test (*blocking of haemagglutination inhibition*) *We have decided to use* this designation for the test which determines the amount of host antigen capable of combining with and thus neutralizing the HI antibody produced against host material We think that the term "blocking" (16) is better than "neutralizing" (8) The *HIB* test *is analogous to the ACHI test (2) In earlier papers* the HIB test has been described in detail (8 15) Here shall only be mentioned that the exact virus and serum doses have to be determined most carefully before each HIB titration, because even a minor change in *e g* the serum dose produces considerable deviations in the *HIB titres Moreover there is some difference* between the sera with regard to the HIB titres given Hence a reference preparation of allantoic fluid is included with every HIB titration and preparations which are to be compared are always titrated simultaneously

The authors are indebted to Mr *A Bye Hansen* Mrs *B Egelandsdal* Miss *T Fotland* and Mrs *I Wang* for skilled technical assistance

Host antigen The main source of chick allantoic fluid was a high speed centrifuge
... production The authors are indebted to Dr *E*

made immediately and after ½ 1 2 and 3 hours

Agar precipitation by the *Ouchterlony* method (13) was performed in plastic

sequently applied on a column Effluent fractions were analysed for HIB activity and ring test precipitation and chemically for neutral sugars, uronic acids, hexosamines proteins and chlorides

Gel filtration was achieved by means of Sephadex G-25 G 100 G 200 (A/B Pharmacia) and Bio Gel P 300 (Bio-Rad/Calbiochem) prepared according to the manu

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distilled water and
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was 1 7

uronic acid with the omission of the orcinol (and in Molisch the alpha napthol) reagent No interfering colour was recorded The orcinol values thus seemed to be fairly representative for the total neutral sugar content under the experimental con

e acetyl
greatly

ysteine sulphuric acid method of *Dische &*

sulphuric acid method (5) and *sialic acids*

Ester sulphate was assayed according to *Dodgson & Price* (6) with potassium

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realized that at least three groups of contaminants (a, b, c) were present in the crude preparation

a) Proteins, giving a precipitation line in agar and ring test precipitation to high titres, but no HIB activity These proteins were almost completely removed on elution at low molarity, i e below 0 30 M NaCl,

which could be separated with 0 45 M NaCl

c) A sugar component, which was quickly released when the eluant molarity was increased above 0 45 The ratio between HIB titre and orcinol value was lower in the first fractions above 0 45 than in later fractions, indicating the presence of contaminating sugar in the first fractions In order to remove this sugar, the elution was carried out with 0.50 M NaCl before collecting fractions for chemical analysis of the HIB antigen —*Elution with 0 45 and with 0 50 M NaCl inevitably lead to a* considerable loss of HIB active material

At higher molarity than 0 50 the host antigen was released with a constant ratio between HIB titre, ring test titre, orcinol and hexosamine values, and it was devoid of uronic acids The recovery of this purified material was 50 per cent, when volumes and HIB titres before and after chromatography were compared

In the present experiments the main problem has been to remove all of the contaminating polysaccharides with the smallest possible loss of HIB active material Thus in one experiment groups of fractions were

collected after raising the eluant molarity above 0 50

Preparative Data of a Large Batch of HIB Antigen

By evaporation under reduced pressure produced by a tap water suction pump 210 litres of centrifuged chick allantoic fluid was reduced to 7 5 litres During and after this operation the fluid was dialysed against tap water and spun, and the sediment was discarded To the concentrated fluid was added 1 5 litre of 0 5 M acetate buffer pH 3 5 to give a final pH of 4 0 This took place at 4° C, and so did the subsequent spinning of the fluid for 1 hour at 2000 r p m The sediment was vigorously shaken for ½ hour at room temperature with 0 4 litre of 0 2 M sodium acetate, and the mixture spun for 1 hour at 5000 r p m The supernate was brownish, opalescent, of pH 5 5 It was subjected to fractionated ethanol precipitation by addition of 280 ml of 96 per cent ethanol, spinning, discarding the sediment and adding 320 ml of the ethanol to the supernatant The last sediment was spun down, and dis-

sulphate as standard Weak hydrolysis with 0 04 N *HCl at 100° C*, sufficient to release the N sulphate of heparin, failed to split off sulphate from our substance Maximal values were reached with 1 N HCl for 5 *hours at 100° C*

Proteins were determined by the Folin Ciolcalteu phenol method according to *Lowry et al* (11) with bovine serum albumin as standard The purified sample was also examined by the micro Kjeldahl method (9)

Chlorides were titrated with silver nitrate using potassium chromate as indicator

EXPERIMENTAL AND RESULTS

Principal Lines

All purification procedures were checked by testing the specific activity expressed by the ratio HIB titre protein (Folin) content The recovery figures with regard to HIB activity were also determined

Purification was accomplished through 3 successive steps

1 Precipitation of the substance at pH 4, and subsequent extractions at a higher pH
2 Fractionated ethanol precipitation
3 Ion exchange chromatography on DEAE Sephadex

Each step will be reported in more detail The optimal conditions for the precipitations were determined for each batch in pilot experiments In order to obtain sufficient amounts of material for chemical analysis a large volume (about 200 l) of allantoic fluid was fractionated

1 Primarily the allantoic fluid (normal fluid or supernate from influenza vaccine production) was concentrated about 30 times by boiling under reduced pressure at 25° C, and subjected to dialysis against tap water The pH was then adjusted to about 4 0 with 0 5 M acetate buffer of pH 3 5 The HIB active material was obtained from the precipitate by extraction with 0 2 M sodium acetate or acetate buffer to give a final pH of 5 4 to 5 8 The specific activity was by these procedures increased between 6 and 30 times with about 50 per cent recovery of HIB activity Moreover, the volume could be reduced 20 times

2 To one volume of the resulting extract, given a pH of about 5 4, was added 0 7 volume of 96 per cent ethanol under constant stirring at +4° C The precipitate was spun down after one hour and discarded Another 0 8 volume of the ethanol (to give a final volume of 1 5) was added to the supernate and left overnight at 4° C The resulting precipitate was dissolved in distilled water The fractionated precipitation with ethanol yielded a further 2 to 4 fold increase of specific activity and 25 to 75 per cent recovery This crude preparation had still a rather high content of proteins It also produced several precipitation lines in agar, thus requiring further purification, which was performed by means of ion exchange chromatography

3 Some preliminary experiments were carried out with Dowex-1, but DEAE Sephadex was preferred because of better resolution of the active material A series of pilot experiments were performed, trying

both gradient and stepwise elution with sodium chloride The fractions were tested serologically and chemically as described above, *and it* was realized that at least three groups of contaminants (a, b, c) were present in the crude preparation

a) Proteins giving a precipitation line in agar and ring test precipitation to high titres, but no HIB activity These proteins were almost completely removed on elution at low molarity, i e below *0 30 M NaCl*, while the host antigen was retained

b) A uronic acid amino sugar component, which could be separated from the host antigen by prolonged elution with 0 45 M NaCl

c) A sugar component, which was quickly released when the eluant molarity was increased above 0 45 The ratio between HIB titre and orcinol value was lower in the first fractions above 0 45 than in later fractions, indicating the presence of contaminating sugar in the first fractions In order to remove this sugar, the elution was carried out with 0 50 M NaCl before collecting fractions for chemical analysis of the HIB antigen —Elution with *0 45* and with *0 50* M NaCl inevitably lead to a *considerable* loss of HIB active material

At higher molarity than 0 50 the host antigen was released with a constant ratio between HIB titre, ring test titre, orcinol and hexosamine values, and it was devoid of uronic acids The recovery of this purified material was 50 per cent, when volumes and HIB titres before and after *chromatography* were compared

In the present experiments the main problem has been to remove all of the contaminating polysaccharides with the smallest possible loss of *HIB* active material Thus in one experiment groups of fractions were concentrated by freezedrying, and subsequent analyses then revealed *a prolonged release of small amounts of contaminating polysaccharides* This made re chromatography necessary for the first *group of fractions* collected after raising the eluant molarity above 0 50

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solved in 400 ml of distilled water These procedures took place at 4° C The titre values are shown in Table 1

TABLE 1

Results Obtained during the Various Stages of Purification of the HIB Antigen from a Large Batch of Allantoic Fluid a Supernate from the Influenza Vaccine Production

Concentration and purification steps	Volume litres	HIB titres	Recovery of HIB antigen (per cent)	Protein (Folin) microg ml	HIB protein	Neutral sugar (Orcinol) microg ml	HIB/neutral sugar
None (crude allantoic fluid supernate)	210	200	–			–	
Evaporation, dialysis and centrifugation	7 5	5 000	80	10 000	0 5	1 200	4
Precipitation at pH 4 0, followed by extraction at pH 5 5	0 4	60 000	60	10 000	6	2 900	20
Fractionated ethanol precipitation	0 4	40 000	70	2 700	15	2 200	18
Ion exchange chromatography on DEAE-Sephadex	0 03	200 000	50	1 000	200	4 300	47

This solution was applied to a 2 3 × 30 cm DEAE Sephadex column, which had been equilibrated with 0 30 M NaCl in 0 02 M acetate buffer pH 6 2 Fractions of 16 ml were collected The column was eluted with *0 30 M NaCl* in order to remove proteins Nearly 80 per cent of the proteins was removed in these fractions, while practically all carbohydrate material was retained, and the eluate showed no HIB activity The next step was a rise to 0 50 M NaCl Carbohydrate material with *high uronic acid and hexosamine content now appeared in the eluate* together with small amounts of protein and some HIB active material Elution was continued until uronic acid was no longer detectable in the fractions The loss of HIB active material in these fractions, which were discarded, was about 25 per cent

Thereafter the bulk of the HIB active material was released by stepwise elution with 0 6, 0 7, 0 8, 0 9 and 1 0 M NaCl The effluent fractions were combined in the corresponding groups Most of the HIB active material was recovered in the 0 6 and 0 7 M groups of fractions Each group was analysed by the Folin, orcinol and carbazole tests, which showed a trace of uronic acid in the 0 6 M group, and possibly also slightly elevated Folin and orcinol values as compared with the other groups The 0 6 M group was therefore subjected to re-chromatography, and this time the column was first eluted with 0 45 M NaCl, which released some uronic acid and *protein with negligible* loss of HIB active

0 8, 0 9 and 1 0 M groups from the
against distilled water and lyophilized
powdery material, which readily dissolved in water. The
ratio of HIB titre to protein (Folin value) achieved by ion exchange chromatography was 15, and the recovery was about 50 per cent.

TABLE 2

Some Chemical Data of the Purified Host Antigen Preparation

Preparation	Protein (Folin) per cent	Neutral sugar per cent	Hexosamine per cent	Methyl pentose per cent	Ester sulphate per cent	Kjeldahl N per cent
Purified host antigen	7 6	35	22	5 3	8 5	3 4

Tests for uronic acid, pentose, and sialic acid were negative

Some chemical data of the purified material have been presented in Table 2 It appears that the protein or peptide content is low, less than 10 per cent of dry weight when calculated from the Folin value, and about 14 per cent when calculated from the Kjeldahl nitrogen value after subtraction of amino sugar nitrogen The present data account for about 80 per cent of the material, without correction for water uptake during hydrolysis

Essentially the same results were shown by the other batches of supernatant allantoic fluid from influenza vaccine production, and also by a batch prepared from normal allantoic fluid from uninfected eggs

The purified material was passed through columns of Sephadex G-25, G-100, G-200, and Bio-Gel P-300, and was eluted with one single peak, before the appearance of the salts In the analytical centrifuge the material sedimented as a single, symmetrical peak, and the sedimentation coefficient under the experimental conditions was 9 3

Immunological Properties of the Purified Substance

The substance showed an HIB activity down to a dilution of 0 03 microgram dry weight per ml under the standard conditions for the HIB test The test could, however, be made more sensitive by reducing the amounts of viral antigen and immune serum employed, and then as little as 0 003 microgram per ml revealed an HIB activity For the ring test 1 microgram per ml was needed The ratio between HIB titre and ring test titre was repeatedly checked during the fractionations, and it was always the same after removal of the protein contaminants by the final chromatography

On agar precipitation the purified substance gave, against anti chick allantoic serum, a strong precipitation line near the serum basin and

solved in 400 ml of distilled water. These procedures took place at 4° C. The titre values are shown in Table 1

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material free of a ... from the former chromatography, dialysed 0 8, 0 9 and 1 0 M groups from the former chromatography, dialysed against distilled water and lyophilized The yield was 352 mg of a white, powdery material, which readily dissolved in water The increase in the ratio of HIB titre to protein (Folin value) achieved by ion exchange chromatography was 15, and the recovery was about 50 per cent

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unbalanced antigen antibody systems, as it was a constant finding with varying antigen and antibody concentrations At present we do not know whether the two lines are caused by two different antigens, by different antigenic groupings on the same substance or by different antibodies to the same substance

There is reason to believe that influenza virus propagated in the chick allantoic cells is furnished with a host antigen, which is the same as the chick allantoic antigen dealt with in the present paper

Further investigations are in progress in this laboratory on the monosaccharide and amino acid composition, the sulphate linkage, and the antigenic determinant groupings of the host antigen

SUMMARY

A method has been developed for the purification of the chick allantoic antigen which combines with influenza virus haemagglutination inhibiting antibody against normal chick allantoic material Three purification steps were employed *1* Acid precipitation followed by extraction at a higher pH—*2* Fractionated precipitation with ethyl alcohol—3 Ion exchange chromatography

The resulting preparation consisted of neutral sugar, hexosamine and ester sulphate, the latter two were in equimolar amounts No uronic or sialic acids were present The protein content was about 10 per cent The substance was antigenic in rabbits, 1 microgram produced a secondary response of haemagglutination inhibiting antibody *In vitro* the antigenic activity was detected at a dilution of 0 003 microgram per ml

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convex towards the same When concentrated preparations were tested (5 or 10 milligrams/ml), another line was also demonstrable, located about midway between the basins Both lines were still found after heating of the antigen for one hour at 100° C, and they were produced with all of our preparations None of the lines were identical with the strong precipitation line given by the protein fraction of the crude antigen This line did not appear when the crude antigen was preheated as above

The immunogenicity of the purified material was tested in rabbits which had been given various intravenous injections of crude allantoic material some months before, and subsequently left undisturbed in order to let the HIB titres drop Five of these sensitized animals received a single injection of one ml of saline which contained 1 microgram of purified substance, while an identical group of five other animals similarly received 0 2 microgram In the former group (1 microgram) two animals developed a significant (at least 4-fold) HIB titre rise after a week, while in the latter group (0 2 microgram) none showed a significant rise

DISCUSSION

The chick allantoic host antigen of the influenza viruses has been prepared in a seemingly native condition by mild procedures It behaved as a homogeneous substance on gel filtration and on ultracentrifugation

Strandli et al examined chemically a semi-purified material of host antigen, it was presumed to be an acid mucopolysaccharide containing uronic acids (15) The latter component has been removed in the present experiments

The acidic property of our substance as demonstrated by ion exchange chromatography is obviously due to the ester sulphate This acidity has probably facilitated the purification procedures, since apparently no material other than the host antigen was released from the column when the eluant molarity was increased above 0 5 However, the possibility of contamination with other acidic polysaccharides cannot be excluded

The host antigen preparation consists of neutral sugar, hexosamine and sulphate with a small protein moiety The sulphate was not released by weak acidic hydrolysis, which should indicate that it is linked as an ester Hexosamine and ester sulphate occur in about equimolar proportion Being a sulphated mucopolysaccharide without uronic acids, the host antigen shows resemblance to the group of acid mucopolysaccharides named keratosulphates (12) (keratan sulphate, acid heteromucopolysaccharide), but more has to be known about the structure of our substance before it can be classified

The purified preparation produced two precipitation lines in agar They do not represent a simple doubling of lines as may be seen in

different antigenic groupings on the same substance [illegible]
antibodies to the same substance

There is reason to believe that influenza virus propagated in the chick allantoic cells is furnished with a host antigen, which is the same as the chick allantoic antigen dealt with in the present paper

Further investigations are in progress in this laboratory on the monosaccharide and amino acid composition, the sulphate linkage, and the antigenic determinant groupings of the host antigen

SUMMARY

A method has been developed for the purification of the chick allantoic antigen which combines with influenza virus haemagglutination inhibiting antibody against normal chick allantoic material Three purification steps were employed 1 Acid precipitation *followed by* extraction at a higher pH —2 Fractionated precipitation with ethyl alcohol —3 Ion exchange chromatography

The resulting preparation consisted of neutral sugar, hexosamine and ester sulphate, the latter two were in equimolar amounts No uronic or sialic acids were present The protein content was about 10 per cent The substance was antigenic in rabbits, 1 microgram produced a secondary response of haemagglutination inhibiting antibody *In vitro* the antigenic activity was detected at a dilution of 0 003 microgram per ml

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THE INFLUENCE OF STEROID HORMONES AND GROWTH HORMONES ON *HETEROLOGOUS* VIRAL INTERFERENCE IN TISSUE CULTURE

By

V REINICKE

Received 6 iii 65

of another virus different from the first virus -- numerous examples of heterologous viral interference have been described (1, 4, 9, 10, 12 and others), and the phenomenon has been observed with inactivated as well as with fully infectious virus (7, 9, 10, 12)

In 1957 *Isaacs & Lindenmann* (13) showed that inoculation of chorio-allantoic membranes with inactivated influenza virus caused the membranes to produce a virus-interfering substance, interferon, which rendered the cells of the chorioallantoic membrane resistent to many completely *unrelated* viruses This observation together with the finding that also fully infectious virus causes production of interferon (2, 27) led to the hypothesis that heterologous viral interference was mediated through interferon (14, 28), a view point which has proved compatible with several experimental observations (1, 3, 15, 20).

However, several authors (4, 9, 12, 18) have pointed out that infectious virus may create a state of heterologous interference without production of detectable amounts of interferon. In consequence it seems possible that there are at least two different types of heterologous interference one of which is caused by infectious virus particles ("infection interference") and one caused by interferon ("inhibitor interference) (9)

The observation that certain steroid hormones inhibit the production of interferon in tissue culture (5, 23, 24) inspired to the present study on hormonal influence upon heterologous viral interference in a chick embryo fibroblast (c e f) tissue culture system An attempt has been made to correlate the influence of hormones upon production of inter-

The author wishes to thank S *Olesen Larsen*, M A (econ) for performing the statistical analysis and Mrs *B Saugbjerg* for skilled technical assistance

feron (23, 24) and upon heterologous viral ("infection") interference in order to obtain information of the nature of heterologous interference particularly concerning the rôle of interferon in this phenomenon

MATERIALS AND METHODS

Most materials and methods have been described in detail in previous papers (17, 21, 22, 23, 24)

Virus Stocks of influenza A WS virus and Sindbis virus the titres of which were $10^{9.1}$–$10^{9.5}$ EID50/ml and $10^{8.2}$–$10^{8.9}$ p f u./ml, respectively were prepared and stored as previously described

In the present study the tissue cultures were inoculated first with influenza A WS virus and later on with *Sindbis* virus to challenge the interference induced by the influenza virus infection Considerably greater variation of number of plaques was observed when *influenza A WS* pre infected cultures were inoculated with Sindbis virus than when non infected cultures were employed This observation is in agree-

Ho (12) in experiments with influenza virus and

s grown in c e f cultures In several titrations con

idbis virus plaques was seen The figure expressing the *plaque count* should in these instances be estimated as a minimum figure for the number of plaques actually present in the culture

Previous studies in this laboratory with non preinfected cultures showed a Poisson distribution of the number of Sindbis virus plaques (17, 22) Statistical analysis *performed on 28* speratate experiments where pre infection with influenza A WS virus was employed revealed that

$$V\{x\} \sim 3\ 63\ M\{x\}$$

Hence the variance was found to be proportional to the number of plaques encountered

If two plaque titrations each comprising n 'double infected cultures are to be *compared the* identity of the mean plaque counts of the titrations may be tested according to

$$u = \frac{S_1 - S_2}{\sqrt{3\ 63\ (S_1 + S_2)}}$$

where S_1 and S_2 are the sums of the number of plaques obtained in the two titrations For practical purposes it is convient to calculate whether

$$(S_1 - S_2)^2 > (S_1 + S_2) \times 13\ 94$$

in which case the difference $S_1 - S_2$ is significant

Tissue culture Chick embryo fibroblast (c e f) tissue cultures were grown in plastic Petri dishes as previously described in medium 199 containing 5 per cent calf serum 4 per cent chick embryo extract and 0 19 per cent sodium bicarbonate Cultures grown in dishes with a diameter of 8 5 cm were used for interference experiments and cultures grown in dishes with a diameter of 4 5 cm were used for titrations of interferon

Titration of interferon Tissue culture fluids were tested for interferon as previously described (23) employing serial two fold dilutions of the inactivated fluids and 3 or 4 tissue cultures per dilution Interferon titres were expressed as 50 per cent Sindbis plaque inhibition end points If the undiluted fluid was found to be without significant inhibitory capacity (17 22) the fluid was considered to contain no interferon

Hormones (2[illegible]) The following hormones representing main groups of steroid hormones i e

electrolyte bal

Hydrocortisone

d aldosterone,

tradiol 17 benzoate Two different growth
(6 16)
lands of

EXPERIMENTAL

In the first experiment cultures pre treated with hydrocortisone 50 γ ml for 24 hours and untreated control cultures were inoculated with influenza A WS virus at a multiplicity of 0 1 to 0 3 (23, 24) Three hydrocortisone and three control cultures were left uninoculated Following virus adsorption for 1 hour at 36° C the inoculum was removed, the cultures washed with 10 ml of Earle s BSS and replenished with maintenance medium The fluids were harvested and pooled according to groups after 24 hours of incubation at 36° C and subsequently tested for interferon (22, 23 24) After the fluids had been withdrawn duplicate hydrocortisone as well as untreated control cultures were challenged with 0 25 ml amounts of serial ten fold dilutions of Sindbis virus containing 22 220 and 2200 p f u , respectively The cultures which had not been inoculated with influenza virus received 22 p f u Following one hour of virus adsorption at 36° C the cultures were overlayed with 5 ml of agar containing medium The overlaying was, as previously described (22) performed in a dark room with only a minimal amount of light to avoid photodynamic inactivation of Sindbis virus (22, 25, 26) The plaques were read after incubation for 40–48 hours at 36° C in a carbon dioxide incubator Table 1 shows the result of the plaque titrations It appears that 24 hours after inoculation of influenza virus a marked though not complete resistance against Sindbis virus infection had been established in the control cultures It also appears that the hydrocortisone treated cultures had a markedly lower resistance against Sindbis virus infection than the controls A certain degree of interference however, was detectable also in the hydrocortisone treated cultures Testing of the tissue culture fluids removed before Sindbis virus challenge revealed that a small amount of interferon had been produced in the control cultures (the undiluted fluid caused a 50 per cent reduction of plaques) while no interferon was detected in the fluid from the hydrocortisone treated cultures (23) These results thus indicate a correlation between a lowering of the interference and reduction of interferon production

The experiments by *Ho* (12) seemed to indicate that influenza A WS virus was able to induce interference in c e f cultures at a time after virus inoculation when no interferon could be detected This observation together with the previous finding (23) that no interferon was observed in influenza A WS inoculated c e f cultures until between 18 and 24 hours after virus inoculation prompted the subsequent experiment in which the time relationship between development of interference and production of interferon were studied in greater detail Four groups each consisting of six c e f cultures were inoculated as described above

with influenza A WS virus 18, 15, 12 and 9 hours before being challenged with Sindbis virus Three of the cultures of each group were challenged with approximately 600 p f u and the residual 3 cultures were challenged with 6000 p f u The plaques were read after incubation for 48 hours It appears from Table 2 that interference could be detected between 9 and 12 hours after inoculation of influenza virus and that the interference gradually increased with continued incubation The fluids removed from the cultures before challenging with Sindbis virus were tested for interferon and, in accordance with previous results, none was found Cultures were also tested, as previously described (23), for the presence of intracellular interferon Also here no interferon could be detected

TABLE 1

The Influence of 24 Hours Preincubation with 50 γ/ml of Hydrocortisone on Heterologous Interference between Influenza A WS Virus and Sindbis Virus in Chick Embryo Fibroblast Cultures Influenza A WS Virus was Inoculated 24 Hours Prior to Sindbis Virus

	Sindbis virus challenge (p f u)	No of plaques appearing in each culture					
		Hydrocortisone			Control		
Preinfection with	2200	340	270		24	40	
influenza A WS virus	*220*	58	– 56		*0*	*0*	
	22	15	6		0	0	
No preinfection	22	25	18	22	22	17	19

TABLE 2

The Influence of Varying Incubation Periods with Influenza A WS Virus on Development of Heterologous Interference between Influenza A WS Virus and Sindbis Virus in Chick Embryo Fibroblast Cultures

Hours of incubation with influenza A WS virus	No of plaques appearing in each culture when challenged with Sindbis virus	
	600 p f u	6000 p f u
9	233 262 298*	total destruction of cells
12	153 130 111*	numerous plaques with marked confluence
15	7, 0, 12	190 200, 209
18	0, 6 0	23 0 13

* Some confluence of plaques

To test whether hydrocortisone would diminish the degree of inter-

to the first experiment (Table 1) was performed [illegible] cubation for 12 hours instead of 24 hours was employed

It can be seen from Table 3 that this experiment also led to a high degree of interference in the control cultures while the hydrocortisone treated cultures showed considerably less interference In this experiment testing of the tissue culture fluids revealed that no interferon could be detected in control nor in hydrocortisone treated cultures

TABLE 3

to Sindbis virus

	Sindbis virus challenge (p f u)	No of plaques appearing in each culture	
		Hydrocortisone	Control
Preinfection with influenza A WS virus	800	113 – 107	8 – 12
	80	16 19	0 0
	8	2 – 3	0 0
No preinfection	8	7 – 9 – 7	8 – 7 10

TABLE 4

The Influence of 24 Hours Preincubation with Varying Concentrations of Hydrocortisone on Heterologous Interference between Influenza A WS Virus and Sindbis Virus in Chick Embryo Fibroblast Cultures

Preincubation with hydrocortisone (γ/ml) before influenza A WS inoculation	No of plaques appearing in each culture when challenged with Sindbis virus	
	400 p f u	4000 p f u
250	199, 148*	290, 204*
100	140, 152*	295, 225*
50	127, 103	211, 175*
10	109, 103	154 179*
1	31, 33	88, 70
Control (No hydrocortisone)	10, 47	91, 99

* Some confluence of plaques

The following experiment was concerned with the influence of preincubation for 24 hours with varying doses of hydrocortisone upon viral interference in influenza A WS inoculated c e f cultures Incubation for fifteen hours with influenza A WS virus was allowed before the cultures were challenged with 400 or 4000 p f u of Sindbis virus The result can be seen from Table 4 which shows a marked reduction of the viral interference in cultures which had been pre-incubated with quantities ranging between 250 γ/ml and 100 γ/ml of hydrocortisone and a somewhat lower reduction when quantities ranged between 50 γ/ml and 10 γ/ml of hydrocortisone The lower limit for detectable reduction was between 10 γ/ml and 1 γ/ml

with influenza A WS virus 18, 15, 12 and 9 hours before being challenged with Sindbis virus Three of the cultures of each group were challenged with approximately 600 p f u and the residual 3 cultures were challenged with 6000 p f u The plaques were read after incubation for 48 hours It appears from Table 2 that interference could be detected between 9 and 12 hours after inoculation of influenza virus and that the interference gradually increased with continued incubation The fluids removed from the cultures before challenging with Sindbis virus were tested for interferon and, in accordance with previous results none was found Cultures were also tested as previously described (23), for the presence of intracellular interferon Also here no interferon could be detected

TABLE 1

The Influence of 24 Hours Preincubation with 50 γ/ml of Hydrocortisone on Heterologous Interference between Influenza A WS Virus and Sindbis Virus in Chick Embryo Fibroblast Cultures Influenza A WS Virus was Inoculated 24 Hours Prior to Sindbis Virus

	Sindbis virus challenge (p f u)	No of plaques appearing in each culture					
		Hydrocortisone			Control		
Preinfection with influenza A WS virus	2200	340	270		24	40	
	220	58	56		0	0	
	22	15	6		0	0	
No preinfection	22	25	18	22	22	17	19

TABLE 2

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To test whether hydrocortisone would diminish the degree of interference of influenza virus inoculated c e f cultures under experimental conditions where no interferon could be detected an experiment similar to the first experiment (Table 1) was performed Influenza virus incubation for 12 hours instead of 24 hours was employed

It can be seen from Table 3 that this experiment also led to a high degree of interference in the control cultures while the hydrocortisone treated cultures showed considerably less interference In this experiment testing of the tissue culture fluids revealed that no interferon could be detected in control nor in hydrocortisone treated cultures

TABLE 3

[illegible]

	Sindbis virus challenge (p f u)	No of plaques appearing in each culture	
		Hydrocortisone	Control
Preinfection with influenza A WS virus	800	113 – 107	8 – 12
	80	16 19	0 0
	8	2 3	0 – 0
No preinfection	*8*	7 – 9 – 7	8 7 10

TABLE 4

The Influence of 24 Hours Preincubation with Varying Concentrations of Hydrocortisone on Heterologous Interference between Influenza A WS Virus and Sindbis Virus in Chick Embryo Fibroblast Cultures

Preincubation with hydrocortisone (γ ml) before influenza A WS inoculation	No of plaques appearing in each culture when challenged with Sindbis virus	
	400 p f u	4000 p f u
250	199 148*	290 204*
100	140 152*	295 225*
50	127, 103	211 175*
10	109 103	154 179*
1	31, 33	88 70
Control (No hydrocortisone)	10 47	91 99

* Some confluence of plaques

The following experiment was concerned with the influence of pre-incubation for 24 hours with varying doses of hydrocortisone upon viral interference in *influenza A WS inoculated c e f cultures Incubation* for fifteen hours with influenza A WS virus was allowed before the cultures were challenged with 400 or 4000 p f u of Sindbis virus The result can be seen from Table 4 which shows a marked reduction of the viral interference in cultures which had been pre-incubated with quantities ranging between 250 γ/ml and 100 γ/ml of hydrocortisone and a somewhat lower reduction when quantities ranged between 50 γ/ml and 10 γ ml of hydrocortisone The lower limit for detectable reduction was between 10 γ ml and 1 γ ml

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	Sindbis virus challenge (p f u)	No of plaques appearing in each culture					
		Hydrocortisone			Control		
Preinfection with influenza A WS virus	2200	340	270		24	40	
	220	58	56		0	0	
	22	15	6		0	0	
No preinfection	22	25	18	22	22	17	19

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* Some confluence of plaques

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extent, while pre-incubation for 6 hours did not lower the interfering effect of the infection with influenza A virus

The subsequent experiments were performed to investigate the influence of steroid hormones other than hydrocortisone upon viral interference in the present tissue culture system. Metandienonum, d aldosterone, testosterone and oestradiol were used at 50 γ/ml concentrations during a pre incubation period of 24 hours Two growth hormones with an activity of 20 IU/mg and 1 IU/mg, respectively, were included in the investigation The technique employed in these experiments was similar to that described above. The results appear from Tables 6 and 7 It can be seen that metandienonum and testosterone markedly diminished the interference obtained after influenza A WS incubation, while d aldosterone reduced the interference somewhat and oestradiol and growth hormones had no influence upon the interference

DISCUSSION

The possibility that the diminished interference found in the steroid treated cells could be due to a hormone induced change in viral reproduction in these cells should be considered Previous findings (23, 24), however, showing that adsorption, penetration and growth of influenza A WS virus occur in the same manner whether the cells have been treated with hormones or not, would seem to exclude this possibility

The finding that steroid hormones are able to diminish the interference established by influenza virus in c e f cultures between 9, 12 and 18 hours after inoculation as well as to inhibit the production of interferon which normally occurs between 18 and 24 hours after infection (23, 24) poses the problem whether these two effects of the steroid hormones are independent mechanisms of action or they are reflecting two different aspects of the same basic effect, *i e* inhibition of interferon production

This leads further to the question whether heterologous interference always is mediated through interferon (14, 28) or it can result also as a direct action of the virus particles *per se* (9) Considerable experimental evidence seems to support both of these hypotheses (1, 3, 9, 12, 15, 18, 20)

In view of the previous findings *viz* that the decreasing effects of metandienonum and testosterone on the production of interferon was greater than effects of hydrocortisone and d-aldosterone (23, 24) it is an interesting observation that metandienonum and testosterone were the steroid hormones which had the strongest decreasing effect on heterologous interference (Tables 6 and 7) while effects of hydrocortisone and d-aldosterone were less pronounced (Tables 4 and 6).

The fact that the above described effects of hormones on interference took place under experimental conditions where no interferon could be detected (2, 9, 23, 27) does not exclude, however, that small amounts of

TABLE 5

The Influence of Preincubation with 50 γ/ml of Hydrocortisone for Varying Periods of Time on Heterologous Interference between Influenza A WS Virus and Sindbis Virus in Chick Embryo Fibroblast Cultures

Hours of preincubation with hydrocortisone before influenza A WS inoculation	No of plaques appearing in each culture when challenged with Sindbis virus	
	400 p f u	4000 p f u
24	56 55 56	257, 307, 256*
12	10 21 20	256 290 302*
6	0 4	36 51 17
Control (No hydrocortisone)	0, 7	44 55 65

* Some confluence of plaques

TABLE 6

The Influence of 24 Hours Preincubation with 50 γ/ml of Metandienonum d Aldosterone and Growth Hormones on Heterologous Interference between Influenza A WS Virus and Sindbis Virus in Chick Embryo Fibroblast Cultures

Preincubation with hormones before influenza A WS inoculation	No of plaques appearing in each culture when challenged with Sindbis virus					
	100 p f u		1000 p f u		10 000 p f u	
Metandienonum	29	16	140	125	almost complete destruction of cells	
d Aldosterone	12	11	68	75	298	270*
Growth hormone (20 IU/mg)	1 – 2		7	13	90	55
Growth hormone (1 IU/mg)	0	2	8	16	56	76
Control (no hormones)	0	3	8	9	88	48

* Some confluence of plaques

TABLE 7

The Influence of 24 Hours Preincubation with 50 γ/ml of Testosterone and Oestradiol on Heterologous Interference between Influenza A WS Virus and Sindbis Virus in Chick Embryo Fibroblast Cultures

Preincubation with hormones before influenza A WS inoculation	No of plaques appearing in each culture when challenged with Sindbis virus								
	0 p f u			00 p f u			000 p f u		
Testosterone	30	35	23	287	288	324	almost complete destruction of cells		
Oestradiol	6	4	1	52	43	50	255	231 – 227*	
Control (no hormones)	6	3 – 1		69	40	61	300	266	200*

* Some confluence of plaques

A study on the influence of varying pre incubation periods with 50 γ/ml of hydrocortisone upon viral interference in c e f cultures was also performed The technique employed was similar to that described above Table 5 shows that pre-incubation with hydrocortisone for 24 hours as well as for 12 hours depressed the viral interference to almost the same

extent, while pre-incubation for 6 hours did not lower the interfering effect of the infection with influenza A virus

The subsequent experiments were performed to investigate the influence of steroid hormones other than hydrocortisone upon viral interference in the present tissue culture system. Metandienonum, d aldosterone, testosterone and oestradiol were used at 50 γ/ml concentrations during a pre-incubation period of 24 hours Two growth hormones with an activity of 20 IU/mg and 1 IU/mg, respectively, were included in the investigation The technique employed in these experiments was similar to that described above The results appear from Tables 6 and 7 It can be seen that metandienonum and testosterone markedly diminished the interference obtained after influenza A WS incubation, while d-aldosterone reduced the interference somewhat and oestradiol and growth hormones had no influence upon the interference

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This leads further to the question whether heterologous interference always is mediated through interferon (14, 28) or it can result also as a direct action of the virus particles *per se* (9) Considerable experimental evidence seems to support both of these hypotheses (1, 3, 9, 12, 15, 18, 20)

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Hours of preincubation with hydrocortisone before influenza A WS inoculation	No of plaques appearing in each culture when challenged with Sindbis virus	
	400 p f u	4000 p f u
24	56, 55, 56	257, 307, 256*
12	10, 21, 20	256, 290, 302*
6	0, 4	36, 51, 17
Control (No hydrocortisone)	0, 7	44, 55, 65

* Some confluence of plaques.

TABLE 6

The Influence of 24 Hours Preincubation with 50 γ/ml of Metandienonum d-Aldosterone and Growth Hormones on Heterologous Interference between Influenza A WS Virus and Sindbis Virus in Chick Embryo Fibroblast Cultures

Preincubation with hormones before influenza A WS inoculation	No of plaques appearing in each culture when challenged with Sindbis virus		
	100 p f u	1000 p f u	10 000 p f u
Metandienonum	29 16	140 – 125	almost complete destruction of cells
d-Aldosterone	12 – 11	68 75	198 270*
Growth hormone (20 IU/mg)	1 2	7 13	90 53
Growth hormone (1 IU/mg)	0 2	8 16	56 – 76
Control (no hormones)	0 – 3	8 – 9	88 48

* Some confluence of plaques

TABLE 7

The Influence of 24 Hours Preincubation with 50 γ/ml of Testosterone and Oestradiol on Heterologous Interference between Influenza A WS Virus and Sindbis Virus in Chick Embryo Fibroblast Cultures

Preincubation with hormones before influenza A WS inoculation	No of plaques appearing in each culture when challenged with Sindbis virus		
	70 p f u	700 p f u	7000 p f u
Testosterone	30 – 35 23	287 – 288 – 324	almost complete destruction of cells
Oestradiol	6 – 4 – 1	52 – 43 50	255 – 231 – 227*
Control (no hormones)	6 – 3 – 1	69 40 – 61	300 266 – 200*

* Some confluence of plaques

A study on the influence of varying pre-incubation periods with 50 γ/ml of hydrocortisone upon viral interference in c e f cultures was also performed The technique employed was similar to that described above Table 5 shows that pre-incubation with hydrocortisone for 24 hours as well as for 12 hours depressed the viral interference to almost the same

extent, while pre incubation for 6 hours did not lower the interfering effect of the infection with influenza A virus

The subsequent experiments were performed to investigate the *influence of steroid hormones other than hydrocortisone* upon viral interference in the present tissue culture system. Metandienonum, d aldosterone, testosterone and oestradiol were used at 50 γ/ml concentrations during a pre-incubation period of 24 hours Two growth hormones with an activity of 20 IU/mg and 1 IU/mg, respectively, were included in the investigation The technique employed in these experiments was similar to that described above. The results appear from Tables 6 and 7 It can be seen that metandienonum and testosterone markedly diminished the interference obtained after influenza A WS incubation, while d aldosterone reduced the interference somewhat and oestradiol and growth hormones had no influence upon the interference

DISCUSSION

The possibility that the diminished interference found in the steroid treated cells could be due to a hormone induced change in viral reproduction in these cells should be considered Previous findings (23, 24), however, showing that adsorption, penetration and growth of influenza A WS virus occur in the same manner whether the cells have been treated with hormones or not, would seem to exclude this possibility

The finding that steroid hormones are able to diminish the interference established by *influenza* virus *in c e f* cultures between 9, 12 and 18 hours after inoculation as well as to inhibit the production of interferon which normally occurs between 18 and 24 hours after infection (23, 24) poses the problem whether these two effects of the steroid hormones are independent mechanisms of action or they are

e inhibition

interference

always is mediated through interferon (14, 28) or it can result also as a direct action of the virus particles *per se* (9) Considerable experimental evidence seems to support both of these hypotheses (1, 3, 9, 12, 15, 18, 20)

In view of the previous findings *viz* that the decreasing effects of metandienonum and testosterone on the production of interferon was greater than effects of hydrocortisone and d aldosterone (23, 24) it is an interesting observation that metandienonum and testosterone were the steroid hormones which had the strongest decreasing effect on heterologous interference (Tables 6 and 7) while effects of hydrocortisone and d aldosterone were less pronounced (Tables 4 and 6).

The fact that the above described effects of hormones on interference took place under experimental conditions where no interferon could be detected (2, 9, 23, 27) does not exclude, however, that small amounts of

tightly cell bound interferon might have been present and that this interferon might cause the state of heterologous "infection" interference observed in the present tissue culture system

It can therefore not be excluded that the effect of steroid hormones on heterologous interference are related to and possibly identical with the *effect of steroid hormones on interferon production* and that the reduced heterologous interference in fact is due to a diminished production of interferon Also the finding that growth hormones were without influence upon heterologous interference as well as upon production of interferon is compatible with this hypothesis

Friedman (8) has suggested a similar hypothesis based upon the inhibiting effect of actinomycin D on heterologous interference as well as on interferon production in c e f cultures inoculated with Chikungunya virus

The previous finding, however, that production of interferon decreased to the same extent by oestradiol as by d-aldosterone (24) while in the present experiments oestradiol in contradiction to d-aldosterone, showed no effect on heterologous interference (Table 7) seem to indicate two separate mechanisms of action for oestradiol thus supporting the hypothesis that two types of heterologous interference exist

Also the finding (Table 5) that pre-incubation with hydrocortisone for 12 hours was sufficient to diminish the heterologous interference while it has been shown previously (23) that pre incubation with hydrocortisone for more than 12 hours was required to decrease interferon production, would seem to indicate the existence of two separate types of heterologous interference

Thus the present experiments have given evidence compatible with the theories that "infection" interference as well as 'inhibitor" interference exist If the theory that very small amounts of interferon might be the cause of "infection" interference is left out of consideration most of the experiments seem to indicate the existence of a virus particle induced heterologous interference susceptible to inhibition by certain steroid hormones

SUMMARY

The effect of pre-incubation for 24 hours with 50 γ/ml of steroid hormones and growth hormones on heterologous viral interference in influenza A WS inoculated chick embryo fibroblast tissue cultures was studied

Under experimental conditions where no interferon could be detected metandienonum, testosterone, hydrocortisone and d-aldosterone were found to reduce heterologous interference against Sindbis virus

Metandienonum and testosterone were observed to have a stronger decreasing effect on interference than hydrocortisone and d-aldosterone while oestradiol and growth hormones had no influence on the degree of heterologous interference

The correlation between the effect of *steroid hormones on hetero logous interference* and the effect of steroid hormones on production of *interferon* is discussed in relation to hypotheses concerning the role of interferon in the phenomenon of heterologous interference

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THE INFLUENCE OF STEROID HORMONES AND GROWTH HORMONES ON THE PRODUCTION OF INFLUENZA VIRUS AND INTERFERON IN TISSUE CULTURE

2 *The Influence of Metandienonum, d Aldosterone, Testosterone, Oestradiol and Growth Hormones*

By

V REINICKE

Received 6 iii 65

In a preceding paper the influence of hydrocortisone, a catabolic steroid hormone, on production of influenza virus and interferon in chick embryo fibroblast (c e f) tissue cultures was described (16)

The present report is concerned with the influence of steroid hormones other than hydrocortisone in c e f tissue cultures Representatives of the main groups of steroid hormones, *i e* anabolic steroids, steroids with primary effect on electrolyte balance, androgenic steroids and oestrogenic steroids were tested in attempts to demonstrate a possible correlation between the effect of these hormones *in vivo* and *in vitro* Two different growth hormones, like certain steroid hormones known to increase protein synthesis *in vivo* (3, 8) were included in the study

MATERIALS AND METHODS

Materials and methods were essentially the same as those described in detail in previous papers (15 16)

The following hormones were employed (15) d Aldosterone 17 α methyl 17 β hydroxy andosta 1 4 dien 3 on—metandienonum (MEN) testosteroneisobutyrate oestradiol 17 benzoate and growth *hormones* obtained from pituitary glands of sheep and swine The growth hormones had an activity of 20 IU/mg and 1 IU/mg respectively

EXPERIMENTAL

A series of experiments was performed to study the effect of hormone pre-incubation for 24 hours on the production of interferon in c e f tissue cultures inoculated with influenza A WS virus The technique employed was the same as described previously for hydrocortisone (16) The media were harvested approximately 30 hours after virus inocula-

The author wishes to thank Mrs *B Saugbjerg* for skilled technical assistance

tion at a time when the interferon production in the system has reached its maximum (16) *No attempts* were made to remove the added hormones from the media *before the interferon* assay since previous experiments had shown that *presence of hormones did not influence the effect* of preformed interferon *when assayed in c e f* tissue cultures (15) The results can be seen from *Table 1 It appears that all* the steroid hormones examined inhibited the production *of interferon* although to a varying degree Pre-incubation with metandienonum and testosterone resulted in an inhibition so *marked* that no, or only traces of, interferon could be detected, while pre-*incubation* with *d-aldosterone* and oestradiol reduced the titre of interferon to values *just significantly* below those of the control (10, 15) In contrast, the *growth* hormones employed were without influence upon the titre of interferon (Table 1, Exp 4)

TABLE 1

' Steroid Hormones and Growth
k Embryo Fibroblast Tissue
4 HS Virus

Hormone	Number of plaques (average of three cultures)							Titre of interferon§
	1*	4*	8	16	32	64	128	
Exp 1								
Metandienonum	152 0	151 0	156 0					0
d Aldosterone	13 3	54 0	121 0					5
None (Control)	0	39 7	93 7					7
Exp 2								
Metandienonum			*16 3*	*32 7*	*57 3*			21
None (Control)					*20 3*	39 6	70 0	71
Exp 3								
Testosterone	70 1	95 6		102 3				Trace
Oestradiol				*56 3*		112 1	114 0	16
None (Control)		6 5		36 6		98 5		29
Exp 4								
Growth hormone (20 IU/mg)				*12 3*	*33 0*	*62 7*		30
Growth hormone (1 IU/mg)				12 1	32 6	63 1		30
None (Control)				11 5	32 0	61 0		30

* Reciprocal of dilutions of media
§ Recorded as reciprocal values

In a subsequent series of experiments the addition of hormones was postponed until three hours after influenza virus inoculation and the production of interferon was again investigated The three-hour-interval was chosen to ensure that virus adsorption and penetration into the cells had taken place (1, 6, 7) before hormone treatment It can be seen form Table2 that also when the hormones were added so late after virus inoculation that a possible influence upon the early phases of infection

could be excluded the steroids inhibited the production of interferon (Table 2 Exp 1 and 2), while growth hormones did not influence the interferon production (Table 2, Exp 3) In accordance with the results obtained in the first experimental series, addition of metandienonum and testosterone resulted in markedly decreased interferon titres, while oestradiol reduced the titer of interferon just significantly below that of the control

TABLE 2

Th
in

Hormone	Number of plaques (average of three cultures)							Titre of interferon §
	2*	4	8	16	32	64	128	
Exp 1								
Metandienonum		16 3	28 7	64 7				11
None (Control)					20 3	40 2	76 8	68
Exp 2								
Testosterone	36 0		116 3		112 6			3
Oestradiol	0 6		28 5		111 6			16
None (Control)			11 3		64 0		117 3	28
Exp 3								
Growth hormone (20 IU mg)				12 0	32 3	62 7		30
Growth hormone (1 IU/mg)				12 2	31 8	60 2		30
None (Control)				11 5	32 0	56 0		30

Reciprocal of dilutions of media
§ Recorded as reciprocal values

In another experimental series studies were carried out concerning the production of influenza A WS virus in c e f cultures which had been either pre treated with hormone or to which hormone was added three hours after virus inoculation The hormone pre treated cultures were inoculated with influenza A WS virus at a multiplicity of 0 1–0 3 Following virus adsorption for one hour at 36° C the inoculum was removed the cultures were washed with 10 ml of Earle s BSS, maintenance medium was added and the incubation was continued at 36° C The untreated control cultures had their medium changed to hormone containing medium three hours after virus inoculation At intervals after virus adsorption duplicate cultures from each of the two experimental series were removed from the incubator together with appropriate control cultures The cells were scraped down and homogenized in the medium by vigorous pipetting and by rapid freezing and thawing three times The cell debris medium mixtures collected during the main experiment were titrated for infectivity and haemagglutinin after storage at —60° C and +4° C respectively (14)

None of the titrations revealed detectable amounts of haemagglutinin and from Table 3 *it appears that the* same infectivity titres of influenza virus were obtained whether the cells had been treated with hormones before or after virus inoculation or *not at all* It can therefore be concluded *that none of the employed* hormones influenced adsorption, penetration or *growth of influenza A WS virus in c e f cultures*

TABLE 3

The Influence of Steroid Hormones and Growth Hormones on the Production of Influenza A WS Virus in Chick Embryo Fibroblast Cultures Either 24 Hours Pre incubation with Hormone or Addition of Hormone Three Hours after Virus Inoculation was Employed

Hormone	Virus titres (I ID₅₀*)			
	Hours after inoculation			
	24		48	
	Pre incub with hormone	Hormone added after inocul	Pre incub with hormone	Hormone added after inocul
Metandienonum	5 1	4 7	4 9	4 1
Testosterone	5 3	4 3	4 7	4 7
d Aldosterone	5 3	4 3	5 1	4 7
Oestradiol	4 7	4 1	4 9	4 5
Growth Hormone (20 IU/mg)	5 3	4 9	4 9	4 7
Growth Hormone (1 IU/mg)	4 7	4 7	4 5	4 1
None (Control)	5 1	4 9	4 9	4 6

* Recorded as $\log_{10}$.

TABLE 4

The Influence of Varying Doses of Metandienonum upon the Production of Interferon in Chick Embryo Fibroblast Cultures Inoculated with Influenza A WS Virus

Amount of metandienonum (γ ml)	Number of plaques (average of three cultures)						Titre of interferon §
	4*	8	16	32	64	128	
50	2 0	42 7	88 0				8
10		1 0	20 0	67 7			23
2			5 0	34 0	79 0		36
0 4			6 0	40 0	79 0		32
0 08			1 7	11 9	54 7		53
0 016				7 3	51 3	83 3	56
None (Control)				10 3	53 0	82 7	54

* Reciprocal of dilutions of media
§ Recorded as reciprocal values

In a final experiment the effect of varying doses of metandienonum upon the production of interferon in influenza A WS virus inoculated c e f cultures was examined Metandienonum concentrations ranging from 0 016 γ/ml to 50 γ/ml were employed The hormone was added three

hours after virus inoculation and the media harvested and pooled after 30 hours incubation (16) and subsequently tested for interferon It appears from Table 4 that the production of interferon increased gradually as the content of metandienonum in the medium was reduced from 50 γ/ml to 0 08 γ/ml It can also be seen that under the experimental conditions employed a minimum of between 0 4 γ/ml and 0 08 γ/ml of metandienonum was required for inhibition of interferon production

DISCUSSION

The present experiments have shown that the steroid hormones metandienonum, testosterone, oestradiol and d aldosterone all inhibited the production of interferon in c e f cultures inoculated with influenza A WS virus The two growth hormones, obtained from sheep and swine respectively (15), were both without influence upon interferon production

The finding that inhibition of interferon production occurred when the cells had been pre treated with steroid hormones as well as when the hormones were added after virus adsorption and penetration had taken place indicates that the inhibition involves intracellular mechanisms and cannot be ascribed to a change in the permeability of the cell membrane influencing virus adsorption and penetration into the cells (16)

Although the steroid hormones examined showed considerable variation as regards the degree of their influence on the interferon production their effect was undoubtedly only inhibitory and probably analogous to the previously deescribed inhibitory action of hydrocortisone on interferon synthesis in chick embryo fibroblasts (16) Thus steroid hormones such as metandienonum and testosterone which are known to enhance protein synthesis *in vivo* (3 8) in the present system markedly reduced the synthesis of a protein *i e* chick interferon Consequently the present experiments have not revealed any correlation between the *in vivo* effects of the hormones and their effect on interferon production in c e f tissue cultures Similar findings have been reported by *De Maeyer & De Maeyer* in tissue culture experiments with rat cells where treatment with metandienonum also was found to reduce the production of interferon (11)

It is an interesting fact that *in vitro* effects diverging from the *in vivo* effects have been described for many hormones (2, 3) For instance it has been shown by *Dierscherl* (4), who studied protein synthesis in mouse liver homogenates that inhibition instead of enhancement of protein synthesis might occur when testosterone, a steroid hormone known to increase protein synthesis *in vivo*, was added to the cell homogenate The rate of protein synthesis was estimated from the rate of incorporation of ^{14}C glycine into proteins and depending on the experimental conditions some evidence was found that rather high doses

of steroid (concentrations in the range of 30 γ/ml to 70 γ/ml) often resulted in inhibition of protein synthesis while smaller concentrations not unfrequently caused enhancement of protein synthesis

This dosage effect leading to either a decrease or an increase of protein synthesis was not encountered in the present experiments As shown in Table 4 metandienonum had only depressive effect on production of interferon within a wide concentration range (50 γ/ml to 0 4 γ/ml) In other words doses ranging from values close to physiological levels of steroid hormones (hydrocortisone (17)) to approximately *200 times these values had principally* the same effect *i e* inhibition of interferon synthesis in the present tissue culture system The effect, however, was more or less pronounced depending on the hormone concentration.

While knowledge of the mechanism of hormone action is still incomplete (5, 9) the difference between the *in vitro* and the *in vivo* studies may well be ascribed to the presence of other hormones in the intact animal It has thus been shown that the anabolic effect of certain steroid hormones is dependent on the endocrinological status of the test animal, *i e* whether the animal for instance is castrated or thyroidectomized (3)

The growth hormones employed in the present study were found to be without effect upon the production of interferon It has been suggested that growth hormones cause enhancement of protein synthesis *in vivo* by stimulating the β-cells of the islets of Langerhans to secrete insulin and that insulin should be the anabolic principle *per se* (8) It might therefore be suggested that the failure of growth hormones to excert any effects on interferon synthesis in the present tissue culture system may be due to lack of insulin

None of the hormones employed had any effect on the production of infectious influence A WS virus in c e f cells (Table 3) The diminished production of interferon in the steroid treated cultures can accordingly not be ascribed to a lower production of influenza virus in these cultures On the other hand, the low level of interferon did not result in an enhanced yield of virus Thus the observation is similar to that described in the preceding paper concerning the effect of hydrocortisone on production of interferon and influenza A WS virus in c e f cells (16) Obviously the steroid hormones employed in the present paper together with hydrocortisone and certain steroid configurated carcinogens (11, 12, 16) have a marked selective influence on the protein synthesizing mechanisms of virus-infected cells decreasing production of the protein interferon without influencing the synthesis of viral proteins (14, 16)

SUMMARY

Pre-incubation with *metandienonum*, testosterone, oestradiol and d-aldosterone at concentrations of 50 γ/ml was found to reduce the

production of interferon in influenza A WS infected chick embryo fibroblast cultures

The degree of the inhibitory effect of the different steroids varied considerably The most marked inhibition was seen when metandienonum and testosterone was employed Addition of the steroid hormones three hours after virus inoculation also decreased the production of interferon proving that the hormones exert their effect at the intracellular level When added after the virus inoculation the minimal inhibitory concentration of metandienonum was found to be between 0 4 γ/ml and 0 08 γ/ml

The same titre level of infectious influenza virus was found whether the cells had been treated with the steroid hormones or not

Growth hormones were found to be without influence upon production of interferon as well as infectious influenza virus

The correlation between the *in vivo* effect on protein synthesis induced by the hormones employed and the *in vitro* effect upon production on influenza virus and interferon in the present tissue culture system is discussed

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SUPPLEMENTA